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The work done in this grant aims at elucidating the function of the *Wnt* signaling molecules in cancer. *Wnt* proteins are secreted and play important roles in the control over growth, in particular in the mammary gland. Wnt genes can also can act as oncogenes in mouse mammary tumors. The work is specifically aimed at identifying a receptor for *Wnt* proteins. The isolation of a receptor is critical to our understanding of the mechanism of action of Wnt proteins. Using genetic and biochemical approaches we wish to identify and to clone the receptor for a *Wnt* gene product in *Drosophila*, called *wingless*. The receptor will be characterized and using the receptor gene, we will subsequently clone mammalian *Wnt* receptors by homology. During the past year, we have accomplished this goal, as a receptor for *wingless* has been identified.

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Introduction

The goal of this grant is to elucidate the function of the *Wnt* signaling molecules in cancer and in early embryogenesis, with emphasis on finding a receptor for *Wnt* proteins. *Wnt* genes encode secreted proteins involved in cell-to-cell signaling. The *Wnt* gene family includes a *Drosophila* gene *wingless*, which genetically has been very well characterized (3, 4). *Wnt* genes are important in growth control, in particular in the mammary gland and, importantly, can act as oncogenes in mouse mammary tumors (4). Problems inherent to the nature of *Wnt* proteins had precluded the isolation and characterization of *Wnt* receptors, which is central to our understanding of their mechanism of action.

Our approach to identify a *Wnt* receptor is two-fold:

1. We use an assay for soluble extracellular *wingless* protein and an *in vitro* cell culture assay to identify *wingless* receptors.

2. In *Drosophila*, we perform genetic screens to identify suppressors and enhancers of an *wingless* phenotype. These modifying genes will be cloned and their properties will be examined by sequencing and transfection.

During the past year, we have succeeded in identifying a protein, D*frizzled*-2, that fulfills the criteria to act as a receptor for *wingless*, and have thereby accomplished the major goal of this grant.

Body

1. *wingless* signaling in vitro; identification of a receptor.

Previously, we had developed an in *vitro* assay for *wingless* signaling in our laboratory, using a cell line (clone 8 or cl-8) derived from *Drosophila* imaginal discs. To measure a response to *wingless*, we examined the expression of the *armadillo* protein, the most ubiquitous downstream genetic target of *wingless* during the development of the fly. Using co-cultured *wingless*-producing S2 cells or the medium from these S2 cells, we found a large increase in *armadillo* levels in the cl-8 target cells. The soluble *wingless* protein has a rapid, concentration-dependent effect and can be depleted by an antibody to *wingless*, providing a quantitative and early effect of an extracellular *Wnt* protein (5). We also found that, in contrast to clone-8 cells, *Drosophila* S2 cells do not respond to the wg protein, indicating that they lack one or more components of Wg signaling (6). This finding suggested a complementation strategy to identify such missing components and we therefore tested whether transfection of receptor candidates would make S2 cells responsive to the *wingless* protein.

One interesting receptor candidate was identified during the characterization of a large family of putative cell surface receptors with extensive homology to the *Drosophila* tissue polarity gene *frizzled* (*fz*). Mutations in *fz* result in aberrant orientations of adult cuticular structures, and the *fz* sequence predicts an encoded protein with a cysteine-rich extracellular domain followed by seven transmembrane segments. Although wg does not appear to be involved in the tissue polarity pathway and *fz* does not appear to be involved in the segment polarity pathway, a possible link between the two pathways is suggested by the requirement in each for the function of dishevelled, another *Drosophila* segment polarity gene.

Based on the sequences of Dfz1 and of three mammalian *fz* genes, degenerate PCR primers were designed for the purpose of amplifying additional *frizzled*-like sequences. PCR amplification using *Drosophila* genomic DNA as a target revealed a novel *frizzled* family member, *Drosophila frizzled* 2 (*Dfz2*). The predicted *Dfz2* protein resembles all other members of the *frizzled* family seven putative transmembrane domains, a cysteine-rich aminoterminal extension and a long intracellular domain.

We examined the time and place of *Dfz2* expression by Northern blot analysis, in situ hybridization, and immunostaining. A 5.5 kb *Dfz2* transcript is found throughout the *Drosophila* life-cycle, most prominently during embryogenesis and late larval and pupal life. A segmental pattern of *Dfz2* expression in early embryogenesis is

reminiscent of the expression patterns of many genes in the segment polarity pathway, including wg. We found a transcript of similar size in *Drosophila* clone-8 cells, a cell line from imaginal discs previously shown to be responsive to Wg activity in vitro. *Drosophila* Schneider 2 (S2) cells, which do not respond to Wg, did not contain detectable *Dfz-2* transcripts.

The absence of *Dfz-2* expression in S2 cells was of interest in view of the lack of response of these cells to Wg (6). We tested a possible function of *Dfz-2* expression in Wg signaling by transfecting an expression construct into S2 cells. In this construct, *Dfz-2* is driven by the metal-inducible metallothionein promoter. In stable cell lines derived after selection in hygromycin, there was a baseline level of expression in cells grown in the absence of inducers, as detected with an antiserum to *Dfz-2* .

We tested a possible function of *Dfz-2* expression in Wg signaling by following the levels of the Arm protein in response to added extracellular Wg protein. In transfected cells, the levels of the Arm protein were similar to those in non transfected cells, irrespective of whether *Dfz-2* expression was elevated by copper induction. When the *Dfz-2* transfected cells were incubated in the presence of soluble Wg protein, the level of the faster migrating (non-phosphorylated) form of Arm protein were increased. The elevation was similar to the response in clone-8 cells elicited by Wg. These results showed that *Dfz-2* acts as a signal transducing molecule for Wg, suggesting that it is a receptor for Wg. To examine binding of Wg to the *Dfz-2* transfected cells, we incubated the *Dfz-2* expressing S2 cells in Wg containing conditioned medium at 4°C, and subsequently stained the cells with an antiserum to Wg. Cells expressing *Dfz-2* stained brightly when incubated with Wg and the antiserum, whereas the controls (non transfected S2 cells or transfected cells without adding Wg protein) showed some spots of background staining. We conclude that the Wg protein can specifically bind to cells expressing *Dfz-2*..

As a test for binding of the Wg protein to *Dfz-2* itself, we constructed a fusion protein containing the cysteine-rich amino-terminal domain of *Dfz-2*, linked to the constant domain of human IgG. We added this fusion protein to the supernatant of metabolically labeled S2 cells producing Wg. The fusion proteins and possible complexes were then retrieved by adding sepharose-ProteinA beads. The *Dfz-2* fusion protein, but not a control Ig, selectively bound to labeled proteins of 52 kD, the size of the mature Wg protein. Normal S2 cells did not produce *Dfz-2* binding proteins.

Hence, we have shown that the *Dfz-2* gene fulfills two criteria to be a receptor for the Wg protein: Wg binds to the *Dfz-2* and binding leads to a biological response; an increase in intracellular Arm concentration. In most vertebrates, more than 10 *Wnt*

genes have been identified. As expected, there exists indeed a large family of fz-like genes in vertebrates, likely candidates for receptors for the other *Wnt* proteins. At this moment, there is no genetic evidence that *Dfz-2* is required for Wg signaling, as no mutants at the gene are available. Possible candidates for *Dfz2* mutant may have arisen from the genetic screen described in 6.2.

This work was done in collaboration with the lab of Dr. Jeremy Nathans, Johns Hopkins University in Baltimore and has been published with acknowledgment of the USAMRMC support (ref 1 in part 8, see appendix).

2. A genetic screen for suppressors of a *wingless* phenotype in *Drosophila*

A second route to the identification of components of *wingless* signal transduction in *Drosophila* is to take advantage of the genetic tools developed in this organism. By performing genetic screens for suppressors of a *wingless*-caused phenotype in the fly, one can uncover mutations in genes that are essential to generate this phenotype. Those genes could encode components of the *wingless* signaling pathway, including the receptor.

We have made several P-element based constructs to obtain ectopic expression of *wingless* in larval imaginal discs, the progenitors of adult tissues. These include a construct in which *wingless* expression is driven by the *sevenless* promoter, pSEW-*wingless*, which is known to be active only in the eye imaginal disc. The transgenic flies that were obtained have a very specific phenotype in the eye: an almost complete absence of interommatidial bristles. This phenotype is 100% penetrant and easy to score with a dissecting microscope. This phenotype is also generated by a *wingless* temperature sensitive allele, but in a temperature dependent manner.

This penetrant adult viable *wingless* phenotype has been used to perform a screen for dominant suppressors or enhancers of *wingless*. The principle behind this screen is to search for mutations that will give a phenotype when one allele has undergone a loss-of-function mutation. Normally, complete absence of one allele will not give a phenotype. But in a genetic background where the phenotype of one gene (in this case *wingless*) is dosage-sensitive, absence of one copy of an interacting gene may modify this phenotype. This screen can be done in the F1 generation. Especially since the phenotype is semi-quantitative (i.e. the number of bristles on the eye can be approximated) this screen is very sensitive to dosage of gene products interacting with

wingless and can identify not only suppressors but also enhancers of the pSEW-*wingless*(ts) phenotype.

We have now isolated approximately 20 suppressors and enhancers of the *wingless* phenotype in the eye. These genes have been mapped and have been assembled into complementation groups. We have also performed clonal analysis of these genes, indicating that some of them also have a phenotype in the homozygous state. Five interesting complementation groups have been found, two of them consisting of known genes.

One is *daughterless* (*da*), a helix-loop-helix protein heterodimerizing with other such proteins and required for neurogenesis. We found that wg expression in the eye reduces the level of *da* expression, the first demonstration of regulation of *da* expression by an extracellular signal.

A second known suppressor is a *Drosophila* tumor suppressor gene, called *warts*. This gene encodes a protein kinase but its biochemical function is not clear. We are currently addressing this by producing antibodies to *warts*.

Among the unknown suppressors is one that maps very close to *Dfz2* (map position 76A). We will directly sequence the *Dfz2* gene in this mutant stock to see if the gene is indeed affected.

Part of this work has been published with acknowledgment of the USAMRMC support (ref 2 in part 8, see appendix)

Conclusions

Since the work started, approximately two years ago, we have made very significant progress. The main goal of the project, the identification of a *wingless* receptor, has been accomplished. We have no *Drosophila* mutants in the receptor gene, *Dfz2*, but we have found a number of suppressor mutations in *Drosophila*, one of which may correspond to the receptor gene. Further work will address the biochemical mechanism of signal transduction by the *Dfz2* receptor, and the interactions between other members of the *frizzled* receptor gene family and the various *Wnt* proteins.

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APPENDIX

A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor

Purnima Bhanot*, Marcel Brink†, Cindy Harryman Samos†, Jen-Chih Hsieh*‡, Yanshu Wang*‡, Jennifer P. Macke*‡, Deborah Andrew§, Jeremy Nathans*‡‖ & Roel Nusse†

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Receptors for Wingless and other signalling molecules of the *Wnt* gene family have yet to be identified. We show here that cultured *Drosophila* cells transfected with a novel member of the *frizzled* gene family in *Drosophila*, *Dfz2*, respond to added Wingless protein by elevating the level of the Armadillo protein. Moreover, Wingless binds to *Drosophila* or human cells expressing *Dfz2*. These data demonstrate that Dfz2 functions as a Wingless receptor, and they imply, in general, that Frizzled proteins are receptors for the Wnt signalling molecules.

There is abundant evidence that secreted Wnt proteins have important signalling functions during animal development. For example, Wnt proteins have been implicated in cell-lineage decisions in *Caenorhabditis elegans*, in embryonic and adult pattern formation in *Drosophila*, in axis formation and dorsal–ventral polarity determination in *Xenopus* embryos, and in central nervous system (CNS) development and oncogenesis in mice[1–3]. However, the Wnt proteins have been difficult to obtain in a soluble form, a problem that has hampered the development of biochemical and cell biological assays. Most information about the mechanism of Wnt signalling has come instead from the genetic analysis of *Drosophila* segment polarity and the role of the *Wnt* gene *wingless* (*wg*; refs 2,4–8). Within each embryonic segment, production of the *wingless* protein (Wg) by a narrow stripe of cells maintains *engrailed* expression in an adjacent stripe of cells.

In the embryonic epidermis the *wg* signalling pathway is defined by several genes: *dishevelled* (*dsh*)[9,10]; *zeste white* 3 (*zw3* or *shaggy*); and *armadillo* (*arm*), a member of the beta-catenin gene family[11], which is thought to be inactivated by *zw3*. The *wg* signal seems to counteract the inhibitory effect of *zw3*, leading to activation of *arm*[12–14]. In *Drosophila* embryos the cytoplasmic levels of the *arm* protein (Arm) are increased as a consequence of *wg* signalling[15]. As judged by sequence data, none of the proteins identified thus far in the signalling pathway is a Wg receptor.

On the basis of genetic interactions between *wg* and other genes in the *wg* pathway, we have established a tissue-culture system for *wg* signalling[16]. In this assay, Wg produced by *Drosophila* S2 cells is added in soluble form to a cell line (clone 8) derived from *Drosophila* imaginal discs[17]. Like *Drosophila* embryos, clone 8 cells respond to Wg by specifically increasing the levels of hypophosphorylated Arm[16], suggesting that these cells express a receptor specific for Wg.

Here we report the identification of a novel *Drosophila* gene, *frizzled2* (*Dfz2*), and demonstrate that it functions as a Wg receptor in cultured cells. *Dfz2* was identified in the course of characterizing a large family of vertebrate and invertebrate homologues of the *Drosophila* gene *frizzled* (*fz*)[18]. Mutations in *fz* result in aberrant orientations of adult cuticular structures, a tissue polarity phenotype[19–21]. The *fz* sequence predicts an encoded protein with an amino-terminal cysteine-rich extracellular domain followed by seven transmembrane segments[22,23]. These characteristics have led to the suggestion that *fz* is a receptor for an unidentified ligand that transmits tissue-polarity information[24]. Although *wg* does not seem to be involved in the tissue-polarity pathway and *fz* does not seem to be involved in the segment-polarity pathway, a possible link between the two pathways is suggested by the requirement in each for *dsh* function[10,25].

Molecular cloning of *Dfz2*

Using the sequences of *fz* and three mammalian *fz* homologues, degenerate polymerase chain reaction (PCR) primers were designed for the purpose of amplifying additional *fz*-like sequences[18]. PCR amplification using *Drosophila* genomic DNA as a target revealed a novel *frizzled* family member, *Drosophila frizzled2*(*Dfz2*). Isolation and sequence analysis of genomic and complementary DNA clones corresponding to *Dfz2* revealed a single coding exon containing an open reading frame of 694 amino acids (Fig. 1*a*). The predicted *Dfz2* protein (*Dfz2*) resembles all other members of the *frizzled* family in having the following structural motifs (beginning at the N terminus): a putative signal sequence, a domain of 120 amino acids with an invariant pattern of ten cysteine residues, a highly divergent region of 40–100 largely hydrophilic amino acids that is predicted to be flexible, and seven putative transmembrane segments (Fig. 1*b*). The C terminus of Dfz2 resembles that of most mammalian frizzled protein in ending with the sequence S/T-X-V. A comparison with all known *frizzled* sequences shows that Dfz2 most closely resembles human fz5 and mouse fz8 with which it shares 49% and 45% amino acid identity, respectively. Fz and Dfz2 share 33% amino-acid identity. The *Dfz2* gene resides at 76A on the polytene chromosome map as determined by *in situ* hybridization (data not shown).

Developmental expression of *Dfz2*

As a first step in elucidating the function of *Dfz2* we examined temporal and spatial expression patterns by northern blot analysis, *in situ* hybridization, and immunostaining. A 5.5-kilobase (kb) Dfz2 transcript is found throughout the *Drosophila* life cycle, most prominently during embryogenesis and in late larval and pupal life (Fig. 1*c*). At 2 hours post-fertilization, embryos have low levels of Dfz2 RNA, which is presumably of maternal origin. *Dfz2* expres-

FIG. 1 Isolation and characterization of *Dfz2*—a novel *frizzled* family member in *Drosophila*. *a*, Alignment of the predicted amino-acid sequences of Dfz2 and Fz. Identical residues are indicated in the consensus and conserved cysteines in the cystein-rich domain[18] are in bold face. *b*, Hydropathy profiles of Dfz2 and Fz proteins were calculated according to ref. 37 with a window size of 15 amino acids; increasing hydrophobicity is downwards. The seven putative transmembrane domains and the signal peptide are indicated by a solid line. c, Expression of *Dfz2* RNA during *Drosophila* development and in clone 8 and S2 cells. Upper panel, a 950-bp segment from the *Dfz2* coding region was used to probe a blot containing 40 μg RNA per track from the indicated stages of *Drosophila* development or 30 μg total RNA from S2 cells and clone 8 cells. For the embryo samples, times during development are indicated in hours. A 5.5-kb transcript was detected at all stages, including 0–2 h post-fertilization. Lower panel, a ribosomal protein 49 (RP49) probe was used to probe the same blot to control for sample loading and transfer efficiency.

METHODS. *D. melanogaster* genomic DNA was used as a template for PCR amplification using fully degenerate primers corresponding to conserved sequences YPERPI (sense) and WFLAA (antisense) as described[18]. A single PCR product corresponding to codons 348–425 of *Dfz2* was obtained and used to screen a *D. melanogaster* genomic DNA library in bacteriophage λ. Sequence analysis of cloned genomic DNA revealed an open reading frame of 694 codons with 33% amino-acid identity to Fz. Partial sequences from 6 independent cDNA clones obtained from a 0–9-h embryo cDNA library (gift from K. Zinn) indicate that the open reading frame is contained within a single exon, that there is at least one 5′ non-coding exon, and that the putative initiator methionine is the 5′-most proximal ATG codon and is located 5 codons 3′ of an in-frame termination codon. The Dfz2 and Fz sequences were aligned using GeneWorks software, and hydropathy profiles were calculated using MacVector 3.5 software. RNA from various stages of *Drosophila* development was isolated by the guanidinium–phenol method[38] or from tissue culture cells by LiCl–urea precipitation[39]. RNA was transferred and hybridized as described[38].

a

```
Dfz2        MRHNRLKVLI LGLVLLLTSC RADGPLHSAD HGMGGMGMGG HGLDASPAPG    50
fz          MWRQILFILP -TLIQGVQRY -DQSPLDASP YYRSGGGL-- --M---ASSG    41
Consensus   M....L..L. ..L..... ....PL.... ....G.G... .........G    50

Dfz2        YGVPAIPKDP NLRCEEITIP MCRGIGYNMT SFPNEMNHET QDEAGLEVHQ   100
fz          TELDGLPHHN --RCEPITIS ICKNIPYNMT IMPNLIGHTK QEEAGLEVHQ    89
Consensus   ......P... ..RCE.ITI. .C..I.YNMT ..PN...H.. Q.EAGLEVHQ   100

Dfz2        FWPLVEIKCS PDLKFFLCSM YTPICLEDYH KPLPVCRSVC ERARSGCAPI   150
fz          FAPLVKIGCS DDLQLFLCSL YVPVC-TILE RPIPPCRSLC ESAR-VCEKL   137
Consensus   F.PLV.I.CS .DL..FLCS. Y.P.C..... .P.P.CRS.C E.AR..C...   150

Dfz2        MQQYSFEWPE RMACEHLPLH GDPDNLCMEQ PSYTEAGSGG SSGGSGGSGS   200
fz          MKTYNFNWPE NLECSKFPVH GGED-LCVAE ------NTTS SASTAATPTR   180
Consensus   M..Y.F.WPE ...C...P.H G..D.LC... .......... S.........   200

Dfz2        GSGSGGKRKQ GGSGSGGSGA GGSSGSTSTK PCRGRNSKNC QNPQGEKASG   250
fz          SVAKVTTRKH ------QTGV ---------- ---------- ESPH--RNIG   202
Consensus   .......RK. ........G. .......... .......... ..P......G   250

Dfz2        KECSCSCRSP LIFLGKEQLL QQQSQMPMMH HPHHWYMNLT VQRIAGVPNC   300
fz          FVC------P V------QL- --KTPLGMG- -----Y-ELK VG-GKDLHDC   229
Consensus   ..C......P .......QL. .......M.. .....Y..L. V........C   300

Dfz2        GIPCKGPFFS NDEKDFAGLW IALWSGLCFC STLMTLTTFI IDTERFKYPE   350
fz          GAPCHAMFFP ERERTVLRYW VGSWAAVCVA SCLFTVLTFL IDSSRFRYPE   279
Consensus   G.PC...FF. ..E.....W ...W...C.. S.L.T..TF. ID..RF.YPE   350

Dfz2        RPIVFLSACY FMVAVGYLS- ---------R N-FLQNEEIA CDGLL--LRE   387
fz          RAIVFLAVCY LVVGCAYVAG LGAGDSVSCR EPFPPPVKLG RLQMMSTITQ   329
Consensus   R.IVFL..CY ..V...Y... .........R ..F..... ..........   400

Dfz2        SSTGPHSCTL VFLLTYFF-G MASSIWWVIL SFTWFLAAGL KWGNEAITKH   436
fz          GHRQTTSCTV LFM-ALYFCC MAAFAWWSCL AFAWFLAAGL KWGHEAIENK   378
Consensus   .....SCT. .F.....F.. MA...WW..L .F.WFLAAGL KWG.EAI...   450

Dfz2        SQYFHLAAWL IPTVQSVAVL LLSAVDGDPI LGICYVGNLN PDHLKTFVLA   486
fz          SHLFHLVAWA VPALQTISVL ALAKVEGDIL SGVCFVGQLD THSLGAFLIL   428
Consensus   S..FHL.AW. .P..Q...VL .L..V.GD.. .G.C.VG.L. ...L..F...   500

Dfz2        PLFVYLVIGT TFLMAGFVSL FRIRSVIKQQ GGVGAGVKAD KLEKLMIRIG   536
fz          PLCIYLSIGA LFLLAGFISL FRIRTVMKTD G-----KRTD KLERLMLRIG   473
Consensus   PL..YL.IG. .FL.AGF.SL FRIR.V.K.. G........D KLE.LM.RIG   550

Dfz2        IFSVLYTVPA TIVIGCYLYE AAYFEDWI-- ------KALA CPCAQVKGPG   578
fz          FFSGLFILPA VGLLGCLFYE YYNFDEWMIQ WHRDICKPFS IPCPAARAPG   523
Consensus   .FS.L...PA ....GC..YE ...F..W... ......K... .PC.....PG   600

Dfz2        K---KPLYSV LMLKYFMALA VGITSGVWIW SGKTLESWRR FWRRLLGAPD   625
fz          SPEARPIFQI FMVKYLCSML VGVTSSVWLY SSKTMVSWRN FVERLQGKEP   573
Consensus   .....P.... .M.KY..... VG.TS.VW.. S.KT..SWR. F..RL.G...   650

Dfz2        RTGANQALIK QRPPIPHPYA GSGMGMPVGS AAGSLLATPY TQAGGASVAS   675
fz          RT-------- -R---AQAYV ---------- ---------- ----------   581
Consensus   RT........ .R......Y. .......... .......... ..........   700

Dfz2        TSHHHLHHHV LKQPAASHV          694
fz          ---------- ---------          581
Consensus   .......... .........          719
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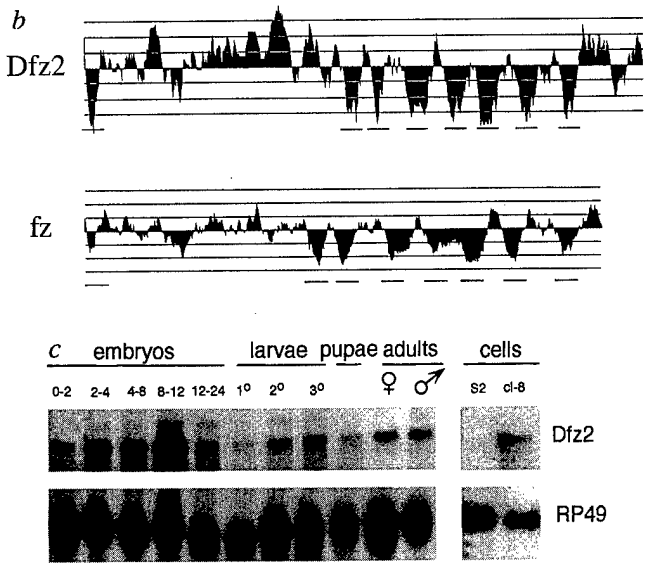


sion is first clearly detected at stage 6, where it is found in all cells between roughly 15% and 70% of egg length, including the invaginating cells of the ventral furrow (Fig. 2*a*). An emerging stripe pattern is evident by early stage 8, and by stage 10 *Dfz2* expression is clearly seen in 15 stripes in the presumptive head and trunk regions, in the posterior midgut primordium, in a subset of cells at the site of anterior midgut invagination, and in the procephalic lobe (Figs 2*b*, *c*). Beginning at stage 12, during germ-band shortening, *Dfz2* expression declines in the epidermis and increases in the midgut and visceral mesoderm (Fig. 2*f*). Beginning at stage 9 and continuing throughout embryogenesis, *Dfz2* expression is also seen in the developing CNS (Fig. 2*d–l*). By stage 17 *Dfz2* expression becomes limited to the CNS, hindgut and dorsal vessel (Fig. 2*l*).

Transfection of *Dfz2* in S2 cells

We also measured Dfz2 RNA and protein levels in clone 8 cells, which respond to added Wg as determined by an increase in Arm protein, and in Schneider (S2) cells, which are unresponsive[16,26]. A *Dfz2* transcript that matches the size of the *in vivo* transcript was observed in clone 8 cells, but no *Dfz2* transcript was detected in S2 cells (Fig. 1*c*). Similarly, in western blots probed with affinity-purified anti-Dfz2 antibodies, a protein band with an apparent mobility of 65K (and comigrating with Dfz2 produced in transfected S2 cells; see below) was observed in samples derived from clone 8 cells but not S2 cells (data not shown). Transcripts derived from *fz* were not detectable in either S2 or clone-8 cells (data not shown).

It is possible that S2 cells fail to respond to added Wg because they do not express *Dfz2*. We tested this directly by transfecting S2 cells with a *Dfz2* expression construct. These cells were then

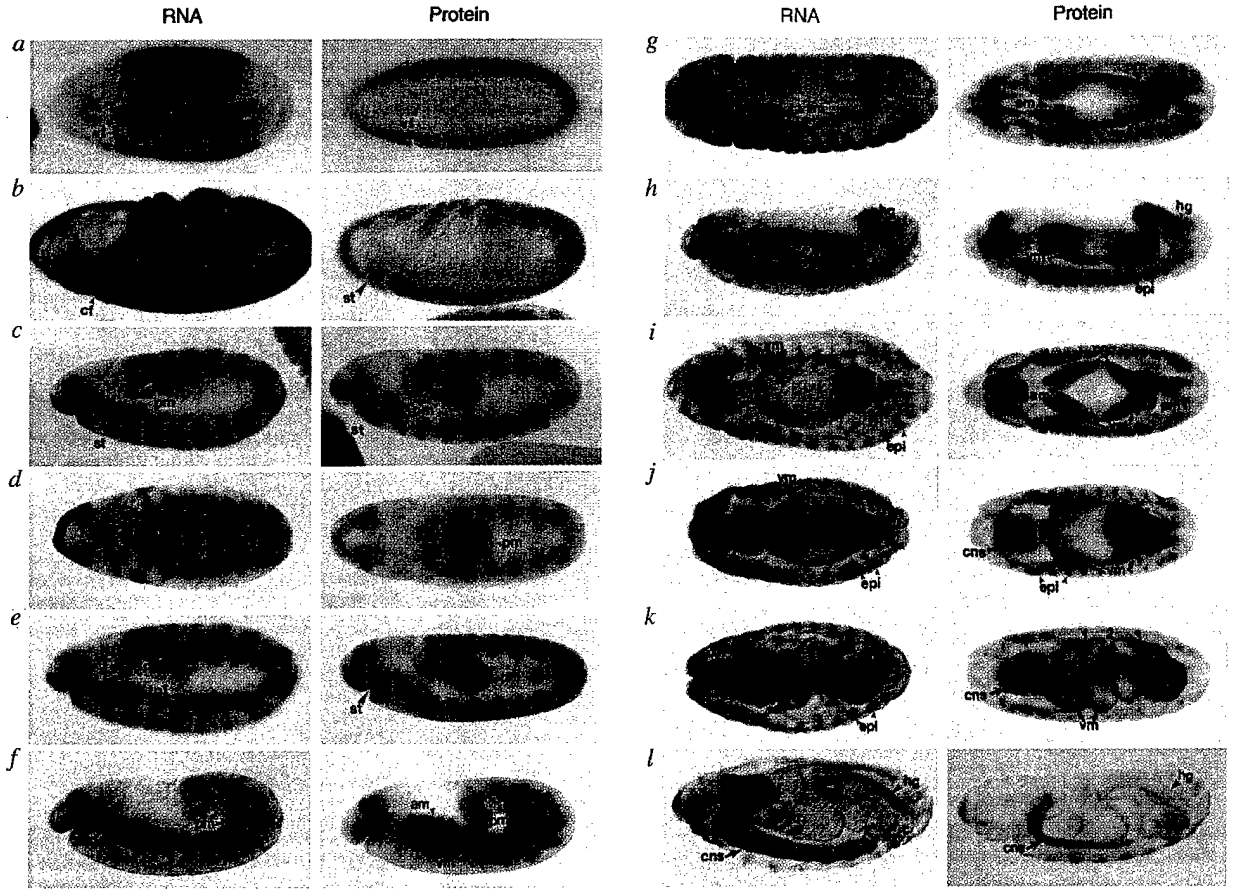


FIG. 2 *Dfz2* RNA and protein in wild-type embryos. Pairs of embryos at the same developmental stage are shown with *in situ* hybridization on the left (blue) and immunostaining on the right (brown). Embryos are oriented with anterior to the left. For embryos shown in a lateral view, dorsal is up. Stages and structures are according to ref. 40. *a*, Stage 6, dorsal/ventral view. *Dfz2* RNA is present in the central region of the embryo and is absent from the anterior and posterior regions. At this stage, Dfz2 protein is below the limit of detection. *b*, Stage 7/8, lateral view. *Dfz2* RNA begins to accumulate in a striped pattern. *c*, Stage 9/10, lateral view. During germ-band extension, *Dfz2* is expressed in 15 stripes in the presumptive head and trunk regions, in the posterior midgut primordium, and in a subset of cells at the site of anterior midgut invagination. *d*, Stage 9/10, dorsal/ventral view. *Dfz2* expression can be seen in the developing CNS. *e*, Stage 10/11, lateral view. *Dfz2* expression resembles stage 9, with transiently higher expression around the primordia of the tracheal pits. *f*, Stage 12, lateral view. During germband retraction, *Dfz2* expression decreases in the epidermis but is maintained at high levels in the anterior and posterior midgut and the presumptive visceral mesoderm. *g*, Stage 13, dorsal/ventral view. The striped pattern of *Dfz2* expression persists in the visceral mesoderm and reappears in cells surrounding the segmental borders. *h*, Stage 13, lateral view. *Dfz2* is expressed at high levels in the hindgut. *i*, Stage 14, dorsal/ventral view. *Dfz2* expression is lower in the anterior and posterior midgut. There is ubiquitous expression in the visceral mesoderm, except in parasegment 7, previously described as the domain of *Ubx* and *dpp* expression[41,42]. *j*, Stage 15, dorsal/ventral view. *Dfz2* is expressed in the CNS, ventral mesoderm, and in cells surrounding the segmental borders. *k*, Stage 16, dorsal/ventral view. *Dfz2* is expressed at high levels in the CNS and in the visceral mesoderm spanning the first midgut constriction and posterior to the second midgut constriction. *l*, Stage 17, lateral view. *Dfz2* expression is primarily in the CNS, with lower levels in the hindgut and the dorsal vessel. Abbreviations: am, anterior midgut; cf, cephalic furrow; cns, central nervous system; epi, epidermis; hg, hindgut; pm, posterior midgut; PS 7, parasegment 7; st, stomodeum; tp, tracheal pit; vf, ventral furrow; vm, visceral mesoderm.

METHODS. Whole-mount embryo *in situ* hybridization was performed on 0–24-h embryos using a digoxygenin-labelled DNA probe encompassing *Dfz2* codons 1–307 as described[43] with minor variations. Fixation was in 4% formaldehyde/1× PBS and the staining reaction was done without levamisole. Identical patterns were obtained with a second probe corresponding to *Dfz2* codons 308–668. Immunochemical localization of Dfz2 protein was done using affinity-purified rabbit antibodies raised against a fusion protein containing the bacteriophage T7 gene-10 protein joined to amino acids 65–314 of Dfz2. Antibodies were purified using a fusion protein containing the *E. coli* maltose-binding protein joined to the same segment of Dfz2. Immunostaining was done as described[44], except that embryos were fixed in Bouin's solution for 30 min instead of 4% formaldehyde/PBS. Antibody staining was visualized by the ABC method (Vector Labs) and embryos were mounted in methyl salicylate.

assayed for the ability to stabilize Arm in response to added Wg. In the absence of Wg, transfected and untransfected S2 cells show similar low levels of Arm irrespective of whether *Dfz2* expression was elevated by copper induction. However, when *Dfz2*-transfected cells were incubated in the presence of Wg, the level of the faster migrating (hypophosphorylated) form of Arm was increased (Fig. 3). This elevation was similar to the response elicited by Wg in clone-8 cells. Increasing *Dfz2* above basal level by copper induction of the metallothionein promoter led to a decrease in Wg responsiveness (Fig. 3), suggesting that at high levels Dfz2 may bind non-productively to second messenger components. Four independent *Dfz2*-transfected cell lines derived from two separate transfections were tested, and all four lines showed Wg-dependent Arm stabilization.

Transfection of *Dfz2* confers Wg binding

The results described above show that *Dfz2* expression confers responsiveness to Wg, consistent with the idea that Dfz2 is a receptor for Wg. To examine Wg binding directly, we incubated *Dfz2*-expressing S2 cells with Wg at 4 °C, and subsequently stained the cells with affinity-purified polyclonal antibodies to Wg. S2 cells expressing *Dfz2* show strong surface staining when incubated with

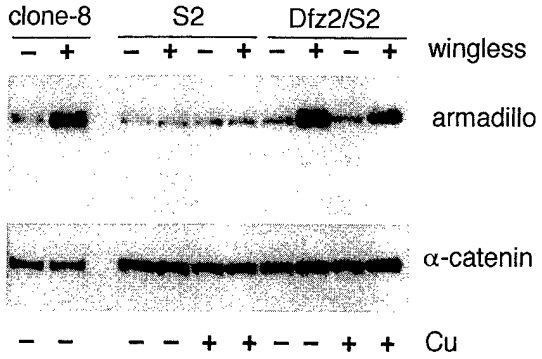


FIG. 3 Addition of soluble Wg leads to an increase in the level of Armadillo protein in *Dfz*2-transfected S2 cells. Clone 8 cells (left), untransfected S2 cells (centre), or *Dfz*2-transfected S2 cells (right) were incubated with concentrated conditioned medium either from S2 cells producing Wg (+Wg) or from control S2 cells (−Wg). Untransfected and *Dfz*2-transfected S2 cells were tested following growth with or without copper sulphate (+Cu or −Cu) to modulate expression of transfected *Dfz*2 from the metallothionein promoter[45]. Cellular proteins were analysed on blots with antibodies against Arm (upper panel). Incubation with Wg-containing medium produces an increase in the level of Arm in clone 8 cells and in *Dfz*2-expressing S2 cells, but not in untransfected S2 cells. Further induction of *Dfz*2 expression in transfected cells by preincubation with copper sulphate leads to a lower response to Wg. As a control for loading, blots were stripped and incubated with antiserum against α-catenin (lower panel).
METHODS. To produce control conditioned medium or conditioned medium containing soluble Wg protein, untransfected S2 cells or S2 cells stably transfected with a construct in which the *wg* coding region is under the control of a heat-shock promoter were used as described[16]. S2 cells stably transfected with *Dfz*2 under the control of the metallothionein promoter were generated by hygromycin selection following transfection with a plasmid carrying the *Dfz*2 coding region inserted into pMK33[45]. Clone 8, S2, and *Dfz*2 transfected S2 cells were incubated with concentrated medium for 2 h. After the incubation, cells were lysed and protein extracts analysed using a monoclonal anti-armadillo antibody 7A1 (ref. 7) or rat-monoclonal anti-α-catenin antibody DCAT-1 (ref. 46). Bound antibody was visualized using the ECL system (Amersham).

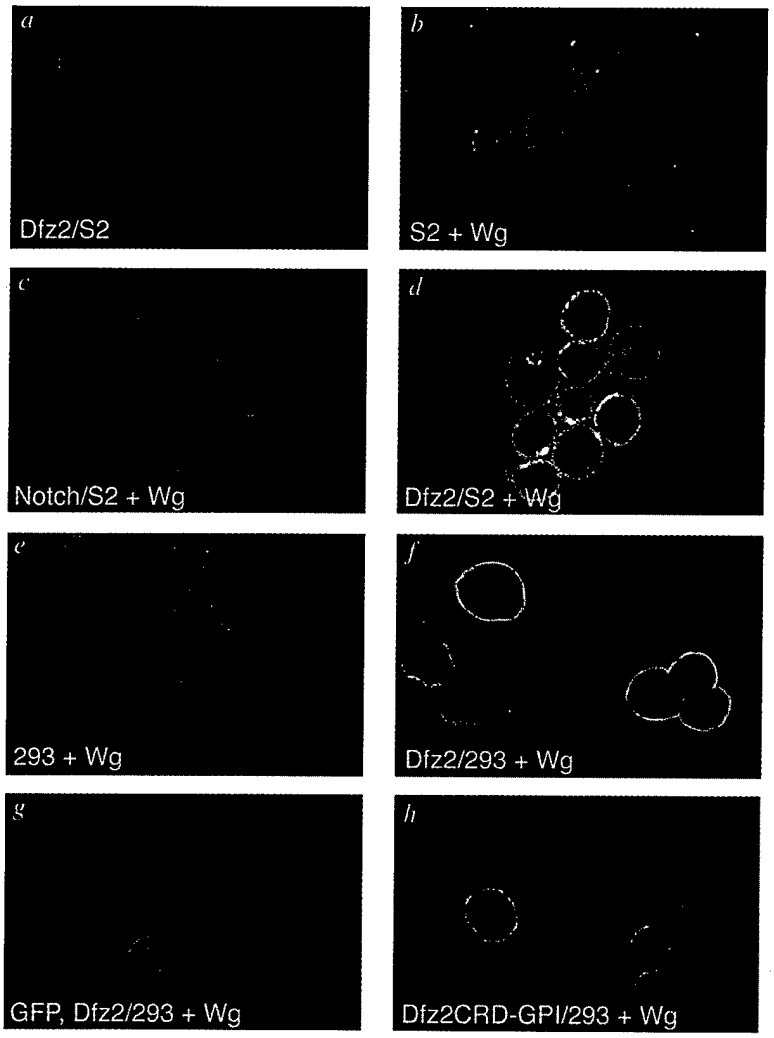


FIG. 4 Wg protein binds to cells transfected with *Dfz*2. Untransfected and transfected cells were incubated with concentrated conditioned medium from untransfected S2 cells (*a*) or from S2 cells producing Wg (*b–h*; see Fig. 3 legend). Following incubation with conditioned medium, the cells were washed, fixed in paraformaldehyde/PBS, and incubated with an anti-Wg antibody directed against an 85-amino-acid domain that is found in Wg but absent from all other Wnt proteins[4]. This domain is dispensable for Wg activity (Chi-hwa Wu, C.H.S. and R.N., unpublished observations). Untransfected S2 cells (*b*), S2 cells transfected with a *Notch* expression plasmid (c), and untransfected 293T cells (human embryonic kidney-cell line 293 stably expressing SV40 TAg; e) show a low level of fluorescent antibody binding. *d*, Roughly 80% of S2 cells stably transfected with *Dfz*2 and incubated with Wg show anti-Wg antibody binding to the cell surface. *f*, 10–20% of 293T cells transiently transfected with a *Dfz*2 expression plasmid and incubated with Wg show anti-Wg antibody binding to the cell surface. *g*, 293T cells cotransfected with a mixture of Dfz2 expression plasmid and a GFP expression plasmid, and incubated with Wg and anti-Wg antibodies show colocalization of green cytosolic fluorescence (GFP) and red surface fluorescence (Wg and anti-Wg antibody) and confirm that 10–20% of cells were transfected, and that only this subset of cells bind Wg. *h*, 293 cells contransfected with a T-antigen expression plasmid and a truncated Dfz2 construct in which the signal sequence, the CRD, and the first half of the linker region are anchored to the cell surface by GPI, show cell-surface binding to Wg and anti-Wg antibody.
METHODS. Untransfected S2 cells and S2 cells expressing *Dfz*2 were washed twice in PBS and incubated with 1.5 ml of 10× concentrated conditioned medium at 4 °C for 3 h. After three 10-min washes with cold PBS the cells were fixed in 2% paraformaldehyde (Polysciences, Inc.) for 15 min at room temperature. After three more 10-min washes with PBS, affinity purified anti-Wg antibody diluted 1:25 in 5% donkey serum/PBS was added to the cells and incubated overnight at 4 °C. After additional washes in PBS, the cells were incubated with fluorescent Cy3 secondary antibody (Jackson Immunoresearch) and mounted. For transient expression in 293T or 293 cells, the *Dfz*2 coding region was inserted into the pCIS expression vector under the control of the cytomegalovirus immediate early promoter/enhancer and with an optimized translation-initiation context, and transfected into 293T or into 293 cells with a T-antigen expression plasmid using the calcium phosphate method[47]. Eight hours after transfection, 10 mM chlorate was added. Twenty-four hours later, the cells were treated with 20 mU of heparatinase (Seikagaku) for 3 h before adding Wg protein. S2 cells expressing Notch[48] were obtained from S. Artavanis-Tsakonas. From N to C-termini, the GPI-anchored construct consists of the first 270 amino acids of Dfz2, a myc epitope[49] and the C-terminal 40 amino acids of decay activating factor, a GPI-anchored protein[50]. Confocal images were collected with a Bio-Rad MRC 1000 confocal laser attached to a Zeiss Axio scope microscope. The same number of scans (20) were taken to visualize the fluorescence of each sample. Images were processed in Adobe Photoshop 3.0.

Wg and anti-Wg antibodies (Fig. 4*d*), whereas *Notch* transfected or non-transfected cells incubated either with or without Wg show a background of randomly distributed spots of low fluorescence intensity (Fig. 4*b*, *c*). *Dfz*2-transfected cells incubated in the absence of Wg show a similar low-intensity, spotty background (Fig. 4*a*). We conclude that Wg specifically binds to S2 cells expressing *Dfz*2.

Although this binding experiment indicates that Wg and Dfz2 probably interact directly, it is possible that expression of *Dfz*2 could act indirectly by inducing or unmasking a Wg receptor. We therefore performed a series of binding experiments using heterologous cells, in this case human embryonic kidney cells (293 or 293T; both will be referred to as 293) and a variety of wild-type and mutant fz constructs. Pretreatment of the 293 cells with chlorate and heparatinase[27] lowered the overall background of Wg binding (presumably binding of Wg to extracellular matrix molecules; Fig. 4*e*), and revealed specific binding of Wg to the surface of 293 cells that had been transiently transfected with *Dfz*2 (Fig. 4*f*) but not to untransfected cells or cells that had been transfected with a bovine rhodopsin expression construct (Fig. 4*e*, and data not shown). In a second experiment in which 293 cells were cotransfected with a green fluorescent protein (GFP) expression plasmid and the *Dfz*2 expression plasmid, we observed that cells with green cytosolic fluorescence (caused by GFP) also had red surface fluorescence (Wg and anti-Wg antibody; Fig. 4*g*).

Each frizzled protein has an extracellular cysteine-rich domain (CRD) that is joined to the transmembrane domain by a variable linker. The CRD has been proposed to constitute part or all of the ligand-binding domain[18], which suggests that cell-surface expression of the isolated CRD segment might confer Wg binding. This possibility was tested by expressing a truncated form of Dfz2 in which the CRD and part of the linker region was displayed on the cell surface as a glycosylphosphotidylinositol (GPI)-anchored protein. This protein was detected at the surface of transfected cells by immunostaining either with antibodies directed against the Dfz2 extracellular domain or with antibodies to a myc epitope tag that was engineered near the C terminus of the GPI-anchored protein (data not shown). When 293 cells transfected with the GPI-anchored Dfz2 CRD were incubated with Wg and anti-Wg antibodies, strong surface staining was observed (Fig. 4*h*). We conclude from this experiment that the Dfz2 CRD constitutes either all, or a significant part of, the ligand-binding domain.

Transfection of a subset of *frizzled* members

In many ligand–receptor systems a single ligand can bind to more than one species of receptor, or a single receptor can bind to more than one species of ligand, or both. Among G-protein-coupled receptors there are many examples of receptor subtypes that recognize the same ligand but differ in effector coupling, tissue distribution and pharmacology. In the fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β/activin/inhibin systems, tissue-culture experiments show that different receptors can bind to a single ligand and that different ligands can bind to a single receptor[28–30].

As a first step in examining the question of ligand–receptor specificity in the Wnt–frizzled system, we tested the ability of Wg to bind to 293 cells transfected with *Drosophila fz* and with six mammalian *frizzled* sequences[18]. 293 cells transfected either with *fz*, human *fz*5 (*Hfz*5), or mouse *fz*4, *fz*7 or *fz*8 (*Mfz*4, *Mfz*7 and *Mfz*8) bind added Wg (Fig. 5*a*), whereas transfection with *Mfz*3 and *Mfz*6 did not confer Wg binding (Fig. 5*b*, *c*). As a complement to the Dfz2 CRD GPI-anchor experiment, a derivative of *Mfz*4 was constructed in which the CRD was replaced with a myc epitope. In transfected 293 cells, immunostaining with an anti-myc antibody, and western blotting with an antibody specific for the *Mfz*4 C terminus show, respectively, that the CRD-deleted Mfz4 protein accumulated at the cell surface and to the same percentage of membrane protein as full-length Mfz4 (Fig. 5*e* and data not shown). However, CRD-deleted Mfz4 did not confer Wg binding (Fig. 5*d*). This experiment further implicates the CRD as an essential determinant of Wg binding. Figure 6 summarizes all of the Wg–frizzled binding experiments described above.

Discussion

The experiments reported here identify a second member of the *frizzled* family in *Drosophila*, *Dfz*2, and show by the following two criteria that it can function as a receptor for Wg. First, transfection of S2 cells with *Dfz*2 confers Wg responsiveness as determined by an increase in cytoplasmic Arm concentration, and second, transfection with *Dfz*2 confers cell-surface binding of Wg in both homologous (S2) and heterologous (293) cell systems. It is important to note that these data do not rule out the possibility that additional molecules in the conditioned medium might associate with Wg and participate in its binding to the receptor. They also do not rule out the possibility that Dfz2 is part of a larger complex at the cell surface; in such a complex Dfz2 would be necessary but may not be sufficient for binding and/or signal transduction. We note that these experiments do not support the proposal that Notch is the Wg receptor[31], because *Notch*-

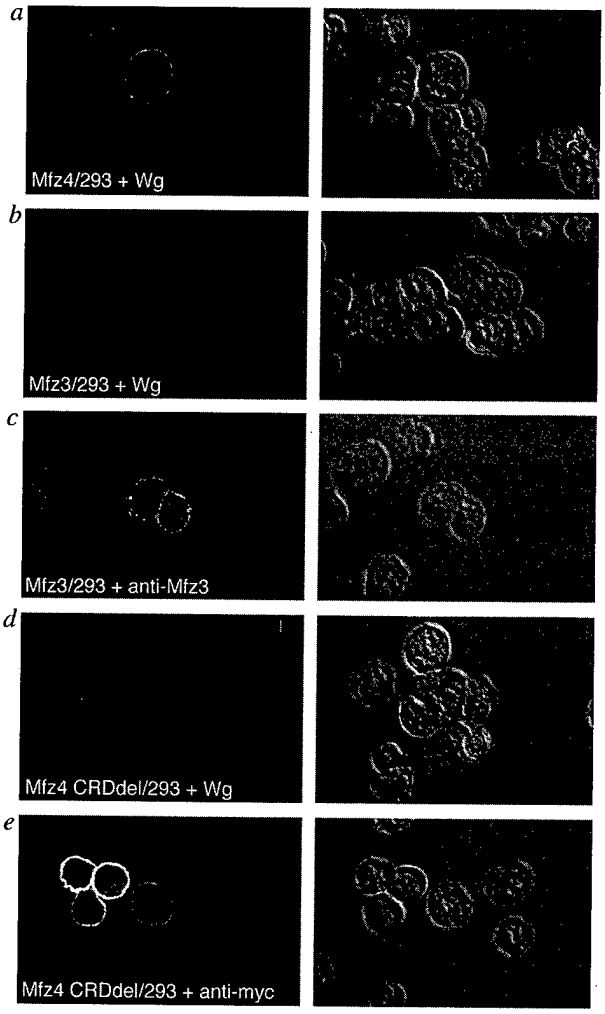
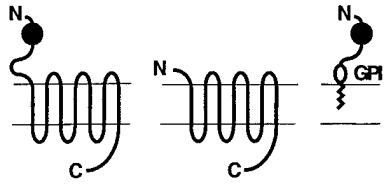


FIG. 5 Binding of Wg to 293 cells transfected with mammalian *frizzled* family members. 293 cells were cotransfected with a T-antigen expression plasmid and the following coding segments inserted into the pCIS vector: *a*, Mfz4; *b*, *c*, Mfz3; *d*, *e*, Mfz4 with the CRD-replaced by a myc epitope. Cells were incubated with Wg and anti-Wg antibodies (*a*, *b*, *d*), affinity-purified antibodies directed against the extracellular domain of Mfz3 (amino acids 1–205; *c*), or anti-myc antibodies (*e*). In each pair of photographs the left hand panel shows the immunostaining and the right hand panel the corresponding phase-contrast image.
METHODS. As described in Fig. 4.



fz	+		
Dfz2	+		+
Mfz3	-		
Mfz4	+	-	
Hfz5	+		
Mfz6	-		
Mfz7	+		
Mfz8	+		

FIG. 6 Summary of Wg–frizzled interactions. Left, intact frizzled protein; centre, frizzled protein with the CRD deleted; right, the frizzled CRD and part of the linker region anchored to the membrane by GPI. + and – indicate the presence or absence of cell-surface binding by Wg after transfection of the frizzled proteins listed on the left. The filled ball represents the CRD. D, *Drosophila*; M, mouse, H, human. Whether Mfz6 is produced and transported to the cell surface has not been determined.

transfected cells do not bind Wg (Fig. 4*c*), nor does Notch confer a Wg-dependent increase in Arm (F. van Leeuwen and R.N., data not shown).

At present, there is no *in vivo* evidence that *Dfz2* is required for Wg signalling, as there are no known *Dfz2* mutants. Although the pattern of *Dfz2* expression is suggestive of its participation in *wg* signalling at multiple points in development, definitive evidence of that participation will require a genetic analysis of *Dfz2* function. The degree to which the *Dfz2* mutant phenotype resembles the *wg* phenotype will most probably depend on whether additional Wg receptors exist *in vivo*.

The ability of Dfz2 to function as a Wg receptor implies more generally that other members of the *Wnt* and *frizzled* families are linked in receptor–ligand relationships. The observation that *Drosophila fz* and some members of the mammalian *frizzled* family also confer Wg binding supports this inference but also suggests that there may be overlapping specificities in Wnt–Frizzled interactions. From the general conclusion that *frizzled* family members encode Wnt receptors, we infer that *in vivo* the *Drosophila fz* protein recognizes at least one Wnt other than Wg (three of which are known[32–34]), and, by extension, that the initial biochemical steps in *fz*-mediated tissue polarity signalling resemble the initial steps of *wg*-mediated segment polarity signalling.

The experiments reported here provide a new point of entry for examining the biochemistry of Wnt signalling. It should now be possible to determine which cytoplasmic proteins interact directly with the frizzled receptors, whether these interactions are modified by Wnt binding, and whether Wnt signalling is regulated by covalent or non-covalent receptor modification. It is interesting to note that many Fz proteins, including Dfz2, contain a S/T-X-V motif at their C-terminal end; this motif has been shown to interact with PDZ (or DHR) domains in a variety of proteins[35]. Dsh, one of the cytoplasmic components of Wg signalling, contains a PDZ domain[9,10].

A question remaining is how frizzled and Wnt proteins might interact to initiate signal transduction. One attractive hypothesis is suggested by the relative immobility of Wnt proteins because of their affinity for the extracellular matrix, and the predicted mobility of the CRD, which we show here constitutes part or all of the ligand-binding site. The prediction that the CRD is mobile follows from the predicted lack of a stable structure in the highly divergent sequence that links it to the membrane-embedded domain. For example, in Dfz2 this linker region includes a stretch of 42 amino acids that includes 21 glycines and 15 serines. Therefore the CRD may be able to bind to an extracellular matrix-associated Wnt protein at a distance of several tens of nanometres from the plasma membrane of the cell on which the frizzled receptor resides. It is tempting to speculate that binding of a Wnt ligand to the CRD disrupts or modifies an interaction between the CRD and the extracellular face of the transmembrane domain, and that this results in a rearrangement of transmembrane α-helices. Although the frizzled proteins have no primary sequence homology to G-protein-coupled receptors[18], this allosteric model suggests a mode of receptor activation that is reminiscent of that proposed for G-protein-coupled receptors[36]. □

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wingless signaling in the *Drosophila* eye and embryonic epidermis

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SUMMARY

After the onset of pupation, sensory organ precursors, the progenitors of the interommatidial bristles, are selected in the developing *Drosophila* eye. We have found that *wingless*, when expressed ectopically in the eye via the *sevenless* promoter, blocks this process. Transgenic eyes have reduced expression of *acheate*, suggesting that *wingless* acts at the level of the proneural genes to block bristle development. This is in contrast to the wing, where *wingless* positively regulates *acheate* to promote bristle formation. The *sevenless* promoter is not active in the acheate-positive cells, indicating that the *wingless* is acting in a paracrine manner. Clonal analysis revealed a requirement for the genes *porcupine*, *dishevelled* and *armadillo* in mediating the *wingless* effect. Overexpression of *zeste white-3* partially blocks the ability of *wingless* to inhibit bristle formation, consistent with the notion that *wingless* acts in opposition to *zeste white-3*. Thus the *wingless* signaling pathway in the eye appears to be very similar to that described in the embryo and wing. The *Notch* gene product has also been suggested to play a role in *wingless* signaling (J. P. Couso and A. M. Martinez Arias (1994) *Cell* 79, 259-72). Because *Notch* has many functions during eye development, including its role in inhibiting bristle formation through the neurogenic pathway, it is difficult to assess the relationship of *Notch* to *wingless* in the eye. However, we present evidence that *wingless* signaling still occurs normally in the complete absence of Notch protein in the embryonic epidermis. Thus, in the simplest model for *wingless* signalling, a direct role for *Notch* is unlikely.

Key words: *wingless*, signal transduction, *Notch*, *Drosophila*, neurogenesis, segment polarity

INTRODUCTION

The *wingless* (*wg*) gene is the best characterized member of the *Wnt* family, which contains over fifty genes in organisms ranging from nematodes to humans (Nusse and Varmus, 1992). *Wnt* genes encode cysteine-rich proteins containing signal sequences and several members, including wg, have rigorously been shown to be secreted (Bradley and Brown, 1990; Fradkin et al., 1995; González et al., 1991; Papkoff and Schryver, 1990; Van den Heuvel et al., 1989; Van Leeuwen et al., 1994).

In *Drosophila melanogaster*, *wg* is required throughout embryogenesis and larval development for a wide range of patterning events (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Some of these include specifying cell fate in the embryonic epidermis (Baker, 1988; Bejsovec and Martinez-Arias, 1991; Dougan and Dinardo, 1992), CNS (Chu-Lagraff and Doe, 1993), mesoderm (Baylies et al., 1995; Wu et al., 1995) and endoderm (Hoppler and Bienz, 1995). In larval development, *wg* is required for patterning in leg (Couso et al., 1993; Diaz-Benjumea and Cohen, 1994; Struhl and Basler, 1993; Wilder and Perrimon, 1995) and wing (Couso et al., 1994; Diaz-Benjumea and Cohen, 1995; Phillips and Whittle, 1993) imaginal discs. In the eye, *wg* has recently been shown to be necessary for proper spacing of morphogenetic furrow initiation (Ma and Moses, 1995; Treisman and Rubin, 1995). How one signal can produce so many responses remains an important unanswered question in developmental biology.

Consistent with being a secreted molecule, wg is thought to execute most of its functions in a paracrine manner. In the best documented cases, the range of wg action can vary from one (Vincent and Lawrence, 1994) to several (Hoppler and Bienz, 1995) cell diameters, though the exact limits of wg diffusion remain unclear (Axelrod et al., 1996; Peifer et al., 1991; Theisen et al., 1994). In a few cases, wg regulates gene expression in the same cells in which it is expressed, e.g. the activation of *cut* expression at the wing margin (Couso et al., 1994) and the regulation of its own expression in the embryo (Bejsovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995). This embryonic autoregulation has been referred to as 'autocrine wg signaling' but it is not clear whether wg works in a truly autocrine manner. However, recent evidence indicates that *wg* autoregulation may have different genetic requirements than the paracrine signaling pathway of wg (Hooper, 1994; Manoukian et al., 1995; see discussion).

Three genes with embryonic phenotypes very similar to that of *wg* have been described (Klingensmith et al., 1989; Peifer and Wieschaus, 1990; Perrimon et al., 1989; Perrimon and Mahowald, 1987), *porcupine* (*porc*), *dishevelled* (*dsh*) and *armadillo* (*arm*). Another gene, *zeste white-3* (*zw3*; also known as *shaggy*) has a mutant phenotype (Perrimon and Smouse, 1989; Siegfried et al., 1992) very similar to that of embryos

where *wg* has been expressed ubiquitously (Noordermeer et al., 1992). Genetic epistasis (Noordermeer et al., 1994; Peifer et al., 1994b; Siegfried et al., 1994) have ordered these genes in the following genetic pathway:

porc → *wg* → *dsh* —| *zw3* —| *arm*

←*porc* has been shown to be involved in either secretion or subsequent diffusion of the wg protein (Siegfried et al., 1994; van den Heuvel et al., 1993a) and the other three genes are thought to be required for receiving the *wg* signal (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994).

Recent work has revealed that many aspects of this embryonic *wg* signaling pathway are conserved in larval *Drosophila* tissues as well as in other organisms. Analysis of *dsh*, *zw3* and *arm* mutations in leg and wing imaginal discs indicates that these genes are required for *wg* signaling (Couso et al., 1994; Diaz-Benjumea and Cohen, 1994; Klingensmith et al., 1994; Peifer et al., 1991; Theisen et al., 1994). This has been best shown in the developing wing margin, where these genes mediate *wg* regulation of the *acheate* (*ac*) gene (Couso et al., 1994; Blair, 1994). The vertebrate homologs of these three genes have been shown to play a role in inducing dorsal mesoderm in *Xenopus* in a manner consistent with functioning in a *Wnt* signaling pathway (Dominguez et al., 1995; He et al., 1995; Heasman et al., 1994; Pierce and Kimelman, 1995; Rothbacher et al., 1995; Sokol et al., 1995).

The *wg* signaling pathway described above was first postulated based on extensive genetic analysis, but recent work indicates that some of the gene products may function directly with wg in a biochemical pathway. The *arm* gene encodes the *Drosophila* homolog of β-catenin (Peifer and Wieschaus, 1990), a component of vertebrate adherens junctions (Kemler, 1993). A similar junctional complex is found in flies (Peifer, 1993) but a substantial pool of cytoplasmic arm protein also exists (Peifer et al., 1994b; Van Leeuwen et al., 1994). *wg* signaling causes an accumulation of cytoplasmic arm protein (Peifer et al., 1994b; Van Leeuwen et al., 1994) caused by a dramatic decrease in arm protein turnover (Van Leeuwen et al., 1994). This accumulation is correlated with a reduction in phosphorylation of arm (Peifer et al., 1994a). This increase in arm protein is thought to somehow transduce the *wg* signal to the nucleus (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994).

Consistent with the proposed genetic pathway, mutations in the other components of the *wg* pathway affect arm protein levels. The normal segmentally repeated accumulation of arm protein is absent in *wg*, *porc* and *dsh* mutants (Peifer et al., 1994b; Riggleman et al., 1990), while *zw3* mutants have uniformly high levels of arm protein (Peifer et al., 1994b; Siegfried et al., 1994). The *dsh* gene encodes a novel protein (Klingensmith et al., 1994; Theisen et al., 1994) containing a PDZ domain (Kennedy, 1995) that is phosphorylated in response to *wg* in embryos and cultured cells, and this phosphorylation is correlated with the ability of *dsh* to stabilize the arm protein (Yanagawa et al., 1995). *zw3* encodes a serine-threonine protein kinase that is homologous with mammalian glycogen synthase kinase-3 (Ruel et al., 1993a; Siegfried et al., 1992). At the present time, it is not clear whether any of the regulatory steps in the pathway are direct or how many missing components remain to be identified.

One new candidate for functioning in the *wg* pathway is the product of the *Notch* (*N*) gene, which encodes a transmembrane protein found on the surface of cells. N protein is thought to act as the receptor for the *Delta* (*Dl*) gene product in a signaling pathway involved in many aspects of development (Muskavitch, 1994; Artavanis-Tsakonas et al. 1995). Its potential role in the *wg* pathway is based on strong genetic interactions between *N* and *wg* mutations in several tissues, but primarily in the wing (Couso and Martinez Arias, 1994; Hing et al., 1994). It is possible that the role of *N* in the separate but oft-used pathway with *Dl* could mask a requirement for *N* in *wg* signaling when *N* mutant embryos or clones are examined. Because N is expressed at the cell surface and appears to act as a receptor, it has been postulated that *wg* encodes a ligand for the N protein (Couso and Martinez Arias, 1994).

This report describes a phenotype created by ectopic expression of *wg* during eye development. These transgenic animals lack the mechanosensory bristles normally surrounding each facet of the compound eye. This is the exact opposite effect seen in the wing, where *wg* is required for bristle formation (Couso et al., 1994; Phillips and Whittle, 1993). Despite this difference in regulation, the *wg* signal transduction machinery found in the embryo and wing also functions in the eye. Finally, the role of *N* in *wg* signaling was examined in the eye and in the embryonic epidermis, where, in the complete absence of N protein, *wg* signaling appears to occur normally. These data argue against a direct role for *N* in *wg* signaling.

MATERIALS AND METHODS

Fly stocks

The mutant alleles in components of the *wg* signaling pathway used in this study were: wg^{IL}, wg^{IN}, wg^{CX4}, $porc^{I8}$, $porc^{2E}$, dsh^{V26}, dsh^{477}, arm^{XM19}, arm^{25B}, $sgg^{D127}$and $zw3^{M11}$. wg^{CX4} (van den Heuvel et al., 1993a,b), dsh^{V26} (Yanagawa et al., 1995) and sgg^{D127} (Ruel et al., 1993b) are null alleles, wg^{IN} encodes a non-secreted wg protein (van den Heuvel et al., 1993a,b), $wg^{IL}$is a temperature-sensitive allele (Baker, 1988) and the rest are characterized phenotypically as strong alleles (Klingensmith, 1993; Siegfried et al., 1992), except for the *arm* alleles, which are hypomorphs but are the strongest alleles that are cell viable when homozygous (Peifer et al., 1991). Two null alleles of *N*, $N^{264.40}$ and N^{5419} (S. Artavanis-Tsakonas, personal comm.) and the temperature-sensitive alleles N^{ts1}(Cagan and Ready, 1989b), Dl^{6E} (Dietrich and Campos-Ortega, 1984) and Dl^{RF} (Parody and Muskavitch, 1993) were also used. For further information, see Lindsley and Zimm (1992).

A P-element construct placing the *wg* ORF under the control of the *sevenless* (*sev*) promoter (P[*sev-wg*]) was made by inserting the *XbaI/ClaI* (blunt ended) fragment of the *wg* cDNA, pCV (Rijsewijk et al., 1987) into the *Xba*I and *Bgl*II (blunt ended) sites of pSEWa (Fortini et al., 1992), between the *sev* proximal promoter and 3′ processing elements. pSEWa also contains three tandem repeats of the *sev* enhancer 5′ of the promoter. yw^{67} embryos were coinjected with P[*sev-wg*] and pπ25.7 as described previously (Rubin and Spradling, 1982) and several independent lines were established using standard balancer stocks. A stock containing the *lacZ* coding sequences under the control of the *sev* enhancer (three tandem repeats) and *hsp70* proximal promoter (P[*sev-lacZ*]; R. Carthew, personal communication) was obtained from Todd Laverty (UC Berkeley, CA).

The following heat-shock strains were used: P[*hs-wg*] (Noordermeer et al., 1992), P[*hs-zw3*] (Siegfried et al., 1992) and P[*hs-dsh*] (Axelrod et al., 1996). P[*hs-wg*] is on the third chromosome, the other two on the second. The following chromosomes were created by recombination. P[*sev-wg*; w^-], P[*hs-zw3*; w^+] (the *white* (*w*) gene in

the P[*sev-wg*; w^+] transgene was inactivated by EMS mutagenesis). A P[*sev-wg*] insert on chromosome 3L was recombined with a Dl^{RF} mutation to make P[*sev-wg*], Dl^{RF}. Two different P[*hs-dsh*; w^+], wg^{IL} recombinants were created, one using a wg^{IL} *cn bw sp* chromosome and the other a wg^{IL} *br pr*, since both chromosomes contain a different lethal mutation unrelated to *wg* (Couso et al., 1994). Both P[*hs-dsh*], wg^{IL} recombinants were placed over a SM5a-TM6B compound chromosome, so that homozygotes could be identified by the absence of the *Tubby* pupal marker.

Whole-mount stainings of pupal eyes and embryos

Pupal eyes were dissected and then immunostained as described (Blochlinger et al., 1993). Embryo stainings were performed essentially as previously described [Frasch et al., 1987; Grossniklaus et al., 1992). Affinity-purified rat α-*cut* antisera was generously provided by K. Blochinger (Fred Hutchinson Institute, WA), mouse α-*ac* monoclonal antibody was a gift of Sean Carroll (University of Wisconsin at Madison). Rabbit α-*lacZ* antisera was from Cappel and affinity purified rabbit α-*wg* antisera was kindly provided by C. Harryman-Samos (Stanford University, CA). Mouse α-*N* monoclonal antibody was provided by S. Artavanis-Tsakonas (Yale Univ. CT) and mouse α-*en* antisera by T. Kornberg (UCSF, CA). The primary antibodies were used at the following dilutions: *ac*, 1:3 to 1:5, *wg*, 1:20, *N*, 1:100, *cut* and *en*, 1:300, *lacZ*, 1:500. For histochemistry, secondary antibodies were either biotinylated (goat α-mouse, horse α-rabbit and rabbit α-rat; all from the Elite ABC kit, Vectastain, used at a 1:500 dilution) or goat α-rabbit conjugated to alkaline phosphatase (from Vector, used at 1:300). For fluorescence microscopy either donkey FITC α-mouse (1:100) or donkey Cy3 α-rabbit (1:200) were used (Jackson Immunochemicals). Confocal images were collected with a Bio-Rad MRC 1000 confocal laser setup attached to a Zeiss Axioscope microscope. Images were imported into Adobe Photoshop for presentation.

In situ hybridization to whole-mount embryos using digoxigenin-labeled probes (Tautz and Pfeiffle, 1989) and antibody/in situ double stainings (Manoukian and Krause, 1992) were performed as described (detailed protocol available upon request).

All whole-mount stainings were photographed with a Nikon Microphot-FXA microscope and slides were scanned into Adobe Photoshop for presentation.

Production of mosaic animals

Mutant alleles of *dsh*, *zw3* and *arm* were recombined unto a P[*hs-neo*; *FRT*]18A chromosome, *porc* onto P[*hs-neo*; *FRT*]19A, *wg* onto P[*hs-neo*; *FRT*]40A and a P[*sev-wg*; w^+] mapping to 3L onto P[*hs-neo*; *FRT*]80A, all in a *w* background. *w* clones were induced in animals heterozygous with the appropriate P[*mini-w*$^+$], P[*FRT*] chromosome: P[*mini-w*$^+$; *hs-πM*]5A, 10D, P[*hs-neo*; *FRT*]18A; P[*mini-w*$^+$]18A, P[*hs-neo*; *FRT*]19A; P[*mini-w*$^+$; *hs-πM*]21C, 36F, P[*hs-neo*; *FRT*]. All FRT derivatives are as described (Xu and Rubin, 1993) except for P[*mini-w*$^+$]18A, which is from the Jan lab enhancer detection collection (Bier et al. 1989). FLP recombinase was provided from the FLP-99 chromosome (Chou and Perrimon, 1992). Clones were induced by a one hour heat shock (37°C) 24-48 hours (at 25°C) after egg laying and scored for the absence of pigmentation in the adult eye.

For production of *N* germ-line clones, the *N* null alleles were recombined onto a P[*mini-w*$^+$; *FRT*]^{101} chromosome (Chou and Perrimon, 1992). *N*, P[*mini-w*$^+$; *FRT*]^{101}/FM7 females were crossed to a w ovo^{D1}, P[*mini-w*$^+$; *FRT*]^{101}/*Y* ; P[*hs-FLP*]^{38} stock (Chou and Perrimon, 1992) and progeny were heat shocked late 3rd instar/early pupation for 2 hours at 37°C (earlier heat shocks resulted in high lethality due to somatic clones). Mosaic mothers were crossed to P[*ftz-lacZ*]C males (Hiromi and Gehring, 1987) or P[*ftz-lacZ*]C; P[*hs-wg*]/TM3 males. Embryos with no β-gal staining lacked both maternal and zygotic expression of *N*.

Heat shocks and other temperature shifts

The P[*hs-wg*] phenotype was induced by multiple heat shocks as previously described (Noordermeer et al., 1992). Late larval/early pupal temperature shifts were performed by submerging glass vials in a water bath of the appropriate temperature (37°C for heat shocks). At all other times, larvae and pupae were kept at 25°C. Formation of white pupae was used as the reference point (0 hours APF).

Histology

Flies were prepared for scanning electron microscopy by serial dehydration in ethanol and Freon 113 (EM Sciences) as described (Kimmel et al., 1990). Dried samples were mounted with colloidal graphite, and a 10 nm gold-platinum coat was applied with a Hummer sputter coater. The samples were viewed with an AMR1000 SEM and photographed using Polapan 400 film (Kodak). Pupal eyes were surface stained with $Co(NO_3)_2{\cdot}6H20$ and $(NH_4)_2S$ as described (Kimmel et al., 1990).

RESULTS

wg blocks SOP formation in the eye

During the course of our attempts to create a dominant adult *wg* mutant through limited misexpression of *wg* during larval development, we found a highly penetrant phenotype when *wg* was placed under the control of the eye-specific promoter *sev*. As shown in Fig. 1, the eyes of P[*sev-wg*] flies appear normal, except that the interommatidial bristles, normally found at alternating vertices in the compound eye's hexagonal array, are almost completely missing. Sections through adult eyes (data not shown) and surface staining of pupal eyes with cobalt sulfide (Fig. 1E,F) revealed no other detectable abnormality in

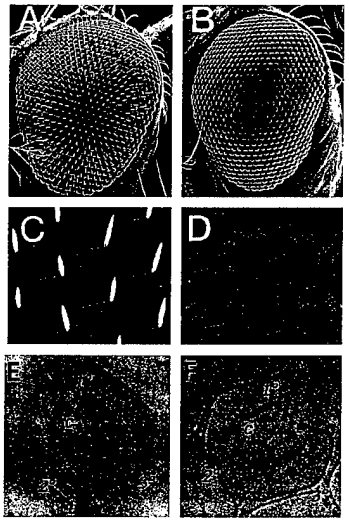

Fig. 1. P[*sev-wg*] flies lack interommatidial bristles. SEM images of parental yw^{67} (A,C) or P[*sev-wg*] (B,D) eyes showing lack of both the base and shaft of the bristles. Note that the hexagonal array and surface of each facet are unaffected in the transgenic eyes. Cobalt sulfide staining of pupal eyes (36 hours APF at 25°C) of control (E) and P[*sev-wg*] (F). Cone cells (c) and the 1°, 2° and 3° pigment cells appear normal in transgenic eyes, but a 3° pigment cell is found in place of each bristle (b).

adult eyes. The bristles are replaced in the repeated structure of the eye with tertiary pigment cells. Thus, at the level of ectopic *wg* expressed from the P[*sev-wg*] transgene, the effect of *wg* on eye development is very specific.

Interommatidial bristles are mechanosensory organs composed of four cells that are derived from a single sensory organ precursor (SOP; (Cagan and Ready, 1989a). Larval SOP determination has been best described in the wing imaginal disc (Campuzano and Modolell, 1992; Jan and Jan, 1993). The process begins with small groups of cells expressing basic helix-loop-helix proteins such as *acheate* (*ac*) and *scute* (Cubas et al., 1991; Skeath and Carroll, 1991). All the cells in these proneural clusters have the ability to become the SOP, however, in a wild-type background, only one does. This cell is thought to become the SOP by reaching a threshold level of *ac* and/or *scute* after which it inhibits these genes' expression in its neighbors (Ghysen et al., 1993; Simpson, 1990). This lateral inhibition is mediated by the neurogenic pathway, in which the products of the *Dl* and *N* genes are thought to act as ligand and receptor, respectively (Artavanis-Tsakonas et al., 1995; Muskavitch, 1994). The initiation of SOP development is correlated with the expression of a new set of genes, such as *neuralized* (Huang and Dambly-Chaudière, 1991) and for some SOPs, *cut* (Blochlinger et al., 1993). The SOP undergoes to two divisions to generate the four cells that will give rise to the mature bristle organ (Bodmer et al., 1989; Hartenstein and Posakony, 1989).

The events leading to SOP formation in the eye have many similarities to those occuring in other tissues. ac protein becomes detectable shortly after white prepupa formation (data not shown). At 3 hours after the white prepupa stage (3 hours APF), the *ac* gene is expressed in small clusters of cells throughout the eye (Fig. 2C). Unlike the photoreceptors and cone cells, the appearance of the ac-positive cells is not related to the distance from the morphogenetic furrow, although the cells anterior of the furrow do not express *ac* (see arrows in Fig. 2C). By 6 hours APF, only one cell per cluster still expresses *ac*, again with the anterior-most portion of the eye showing a less mature pattern (data not shown). At 15 hours APF, after the eye disc everts, ac protein is gone, but the daughters of the SOPs can be observed by staining with α-*cut* antisera (Fig. 2A). Because of the complicated morphogenetic movements associated with the eye/head disc eversion, we have been unable to stain tissue between 6 and 15 hours APF.

In the P[SEV-wg] eyes, *ac* expression is greatly reduced compared to controls though not completely absent (Fig. 2D,F). After disc eversion, no SOPs are found, as judged by *cut* staining (Fig. 2B) and an enhancer detector line for the *neuralized* gene (data not shown). Thus, *wg* appears to act at the level of the proneural genes, i.e., *ac*, to inhibit SOP formation.

wg-dependent SOP inhibition is a paracrine effect

The activity of the *sev* promoter has been well studied in third instar larva, by monitoring endogenous sev expression (Tomlinson et al., 1987) and with chimeric constructs (Bowtell et al., 1989) using *sev* enhancer and promoter elements similar to the ones in P[*sev-wg*]. The enhancer is active in the cone cells and in a subset of the underlying photoreceptor precursors. No description of *sev* expression has been reported after pupation, so the possibility existed that *wg* was expressed in the proneural cells of P[*sev-wg*] eyes, suggesting a possible autocrine effect.

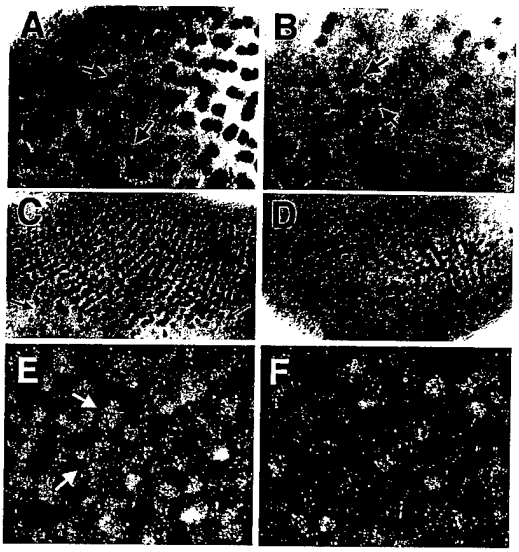

Fig. 2. P[*sev-wg*] eyes have lower than normal levels of ac protein and no SOPs. yw^{67} (A,C,E) and P[*sev-wg*] (B,D,F) pupal eyes were stained with antibodies against cut (A,B; 15 hours APF) or ac (C-F; 3 hours APF). E and F are confocal images. Pairs of cut-positive SOP daughter cells (see arrows) can be seen in controls (A) but not in the transgenic eyes (the arrows point to cone cells, which also express *cut* and lie in a slightly more apical focal plane). *ac* is expressed basally in small clusters up until the morphogenetic furrow (C; arrows indicate the approximate position of the furrow. Anterior is down). The ac-positive clusters usually consist of two or three cells (E; see arrows). ac protein remains in P[*sev-wg*] eyes to varying degrees (the image in D lies in the middle of the range; F shows a close up of an area with relatively high levels of *ac* expression), but staining is always significantly less than controls (E).

This question was addressed by examining the distribution of wg protein in P[*sev-wg*] eyes. Though wg is a secreted protein, it is found at the highest levels on the surface of the same cells that synthesize it (Bejsovec and Wieschaus, 1995; Couso et al., 1994; van den Heuvel et al., 1993). In P[*sev-wg*] eyes, the highest levels of wg protein were found around the four cone cells (Fig. 3A) and accumulated on their apical surface (Fig. 3B). In more basal sections of the eye, wg protein was associated with the photoreceptors, which extend basally to the same plane as the ac-positive cells (Fig. 3C). There was no significant overlap between wg protein and the remaining cells expressing *ac*.

To confirm that the sev enhancer was not active in the proneural clusters, we stained eyes of flies that contained a P[sev-lacZ] transgene (see Materials and Methods) for products of lacZ and ac. As found for wg in P[sev-wg] eyes, most of the β-gal was found in the cone cells (data not shown). In the same focal plane as the ac-expressing cells, there is no overlap (Fig. 3D). Thus, the inhibitory effect of wg on ac expression is paracrine in nature.

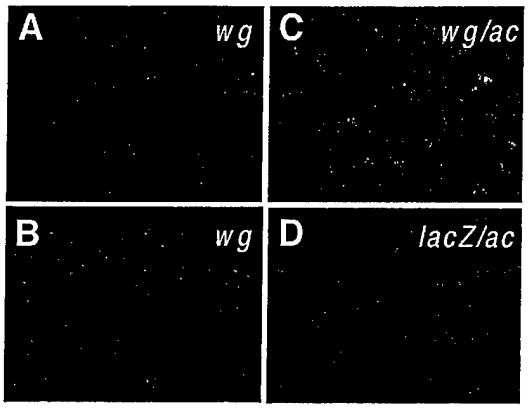

Fig. 3. The *sev* enhancer is not active in the ac-positive cells. P[*sev-wg*] (A-C) or P[*sev-lacZ*] (D) pupal eyes (all at 3 hours APF) were stained with antibodies against wg (A,B), wg and ac (C) or β-gal and ac (D) protein. All panels are confocal images, with *wg* and *lacZ* signals always in red and *ac* always in green. In control eyes, wg protein was detected in a ring around the periphery of the eye (data not shown), but no wg protein was detected in the eye proper. In P[*sev-wg*] eyes, wg is found primarily around the cone cells (A) and accumulated on their apical surface (B) but not in the few remaining ac-positive cells (C). The focal plane in C is about 15-20 μm basal of those in A and B. In the P[*sev-lacZ*] eyes (where *ac* expression is not affected) no β-gal protein is seen in the proneural clusters (D).

The *wg* signal transduction pathway in the eye

Extensive genetic analysis, confirmed by recent biochemical experiments, has identified four genes that encode probable components of the *wg* signaling pathway, *porc*, *dsh*, *zw3* and *arm* (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994; see introduction). Mosaic analysis (using the *w* gene as a marker) was performed to determine if these genes were required for the P[*sev-wg*]-dependent bristle inhibition. Control clones still lack bristles (Fig. 4A), as do clones mutant for the endogenous *wg* gene (Fig. 4C). In clones that lack the P[*sev-wg*] transgene, bristles are found almost to the clonal boundary (Fig. 4B). Likewise, 89% of the mutant clones for *porc*, *dsh* and *arm* had the full array of bristles within the clone (Fig. 4D-F and Table 1) and an additional 9% had a partial rescue of the bristleless phenotype. The remaining 2% that still lacked bristles were small in size and probably not completely mutant since the absence of the *w* gene cannot be detected on the surface of the eye at the cellular level. These experiments indicate that *porc*, *dsh* and *arm* are required for *wg*-dependent bristle inhibition.

zw3 is unique among the known genes required for *wg* signaling because it must be inhibited for the *wg* signal to be transduced (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Thus, loss of *zw3* should be equivalent to activation of *wg* signaling. Therefore, a *zw3* mutant clone in the eye might be expected to lack bristles. This straightforward analysis cannot be employed because the cells in *zw3* clones in the eye imaginal disc do not differentiate into eye tissue (Treisman and Rubin, 1995; data not shown). This is probably due to the fact that high levels of *wg* signaling activity prevent the morphogenetic furrow from progressing, blocking any subsequent differentiation (Treisman and Rubin, 1995).

If *zw3* must be inhibited for the *wg* signal to be transduced, then flooding cells with zw3 protein might titrate out the signal. This has been shown to be the case in *Xenopus* where overexpression of the homologue of zw3, glycogen-synthase kinase 3, blocks *Wnt* gene induction of dorsal mesoderm (Dominguez et al., 1995; He et al., 1995). We attempted a similar experiment by creating flies with one copy of P[*sev-wg*] (we chose one of the weaker P[*sev-wg*] lines, which at one copy has approximately 20 bristles/eye) and one or two copies of a heat-shock construct expressing the *zw3* gene, P[*hs-zw3*] (Siegfried et al., 1992). *zw3* was induced by heat shock shortly before and twice after the onset of pupation (see Fig. 5 legend for details). Though the results were not entirely conclusive (Fig. 5), many pupal eyes showed a significant response especially when the ratio of P[*hs-zw3*]/P[*sev-wg*] is two (Fig. 5C). Other heat-shock regimes were not as effective at suppressing the P[*sev-wg*] phenotype. These results are consistent with the current model for *zw3* function in *wg* signaling.

Overexpression of *dsh* has previously been found to mimic *wg* signaling in cultured cells (Yanagawa et al., 1995), frog embryos (Sokol et al. 1995; Rothbacher et al. 1995) and in the wing imaginal disc (Axelrod et al., 1996). The same P[*hs-dsh*] transgenic stock used in the wing can also duplicate the effect of *wg* in the eye. Induction of *dsh* at 3 hours (data not shown) or 6 hours APF (Fig. 6B) could block bristle formation, but heat shock at 9 hours APF (Fig. 6C) failed to inhibit bristles in the interior of the eye, though inhibition still occurred toward the periphery. This can be explained by previous work

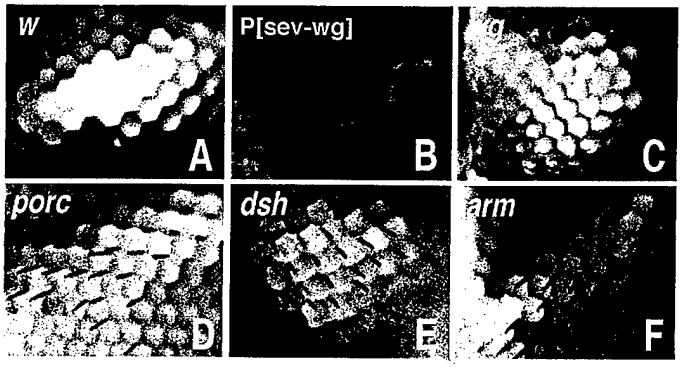

Fig. 4. The *porc*, *dsh* and *arm* genes are required for the P[*sev-wg*] phenotype, but the endogenous *wg* gene is not. Clones were induced in P[*sev-wg*] eyes as described in Materials and Methods. Clones were detected by the absence of pigmentation (from the *w* gene) in adult eyes. Bristles were still absent in control (A) or wg^{CX4} clones (C), but not in clones lacking the transgene (B) or homozygous for *porc* (D), *dsh* (E) and *arm* (F). A summary of all the data can be found in Table 1.

Table 1. Summary of the clonal analysis in a P[sev-wg] background (see Materials and Methods for details)

	Bristle density inside clone		
Chromosome	Bare	Partial	Full
P[*sev-wg*; w^+]	0	1	17
w	25	0	0
yw	21	1	0
wg^{CX4}	23	0	0
yw $porc^{2E}$	0	1	26
yw $porc^{18}$	2	5	25
yw dsh^{477}	0	1	29
yw dsh^{V26}	1	4	14
w arm^{25B}	0	0	15
w arm^{XM19}	0	1	11

The P[*sev-wg*; w^+] clones are *w*;+/+ clones surrounded by *w*; P[*sev-wg*; w^+] tissue. The rest are clones of the homozygous genotype indicated and the entire eye, including the cells in the clone, are P[*sev-wg*; w^-]/+. Bare means no bristles found within the clone and full means the normal wild-type bristle density.

(Cagan and Ready, 1989a,b), which showed that SOP determination occurs first in the center of the eye and radiates outward concentrically. The same time requirements were seen when the bristles were inhibited using P[*hs-wg*] (data not shown).

Genetic and biochemical evidence places *dsh* downstream of *wg* in the signal transduction pathway (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994; Yanagawa et al., 1995), suggesting that the overexpression of *dsh* can bypass *wg* function. However, in the wing, where *dsh* overexpression causes an expansion of the wing margin, it appears that *wg* gene activity is needed to see the *dsh* effect (Axelrod et al., 1996). In the eye, the opposite appears to be true. In pupa homozygous for a *wg* temperature-sensitive mutation, induction of *dsh* after 6 hours at the restrictive temperature still inhibited SOP formation (Fig. 6E). Thus it appears that *dsh* in the eye can act independently of *wg*, though caveats remain (see discussion).

The role of *N* in *wg* signaling in the eye

A strong interaction between mutations in the *N* and *wg* genes has been described (Couso and Martinez Arias, 1994; Hing et al., 1994), which suggests that the two genes have common developmental targets in some tissues. One report suggested that *wg* encodes a ligand for *N*, based on these genetic interactions and the fact that *N* encodes a transmembrane receptor-like protein (Couso and Martinez Arias, 1994). In the eye, *N* activity is required for almost every differentiated cell type (Cagan and Ready, 1989b), so examining *N* clones in a P[*sev-wg*] background was not possible. Therefore, we utilized N^{ts1}, a temperature-sensitive allele (Cagan and Ready, 1989b). When these flies were reared at the restrictive temperature for 3-11 hours APF in a P[*sev-wg*] background, a strong suppression of the *wg* bristleless phenotype was seen (Fig. 7B). This is consistent with a proposed role for *N* in transducing the *wg* signal. However, removal of *Dl* activity for the same time period also suppresses the P[*sev-wg*] phenotype (Fig. 7C). *N* and *Dl* are key components in the lateral inhibition pathway (functioning as receptor and ligand respectively) that insures the proper number of bristles in the eye (Cagan and Ready, 1989b; Parody and Muskavitch, 1993; note the abnormally high bristle density in Fig. 7B and C). This pathway is independent of *wg*, since mutant clones of *wg*, *porc*, *dsh* and *arm* in an otherwise wild-type background have the normal number of bristles (data not shown; see also Fig. 4D-F). Thus, the observation that loss of *Dl* activity can suppress the P[*sev-wg*] phenotype as well if not better than loss of *N* raises the possibility that the interaction between *N* and *wg* in the eye is due to the role of *N* in the lateral inhibition pathway.

If a higher level of *wg* expression is used (via a heat-shock promoter) all the bristles in the N^{ts1} background can be inhibited (data not shown; pupa were placed at the restrictive temperature for 6 hours before a 30 minute heat-shock pulse was given at 6 hours APF). However, it is known that the N^{ts1} allele does not completely remove *N* activity (Couso and Arias, 1994; Hartenstein et al., 1992) so this result is inconclusive. In the eye, it is not possible to determine whether *wg* works

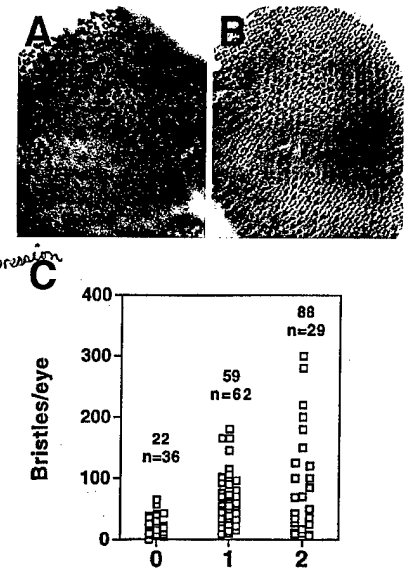

Fig. 5. Overexpression of *zw3* can suppress the P[*sev-wg*] phenotype. Three 1 hour heat shocks (37°C separated by two 4 hour recovery periods at 25°C) were given to animals containing one copy of P[*sev-wg*] and zero, one or two copies of P[*hs-zw3*] (the genotypes of the three groups were P[*sev-wg*; w^+]/+, P[*sev-wg*; w^+]/P[*hs-zw3*; w^+] and P[*sev-wg*; w^-], P[*hs-zw3*; w^+]/P[*hs-zw3*; w^+], respectively; all combinations were created from crosses of stocks described in Materials and Methods). The first heat shock was given at 1-2 hours prior to white pupa formation. An example of a control with about 25 SOPs (A) and a 1× P[*hs-zw3*] eye with about 180 SOPs (B) are shown. SOPs were detected with cut immunostaining. (C) The total data are summarized in a scatter plot. The mean number of SOPs are shown above each group, with the *n* value below. The standard deviation for the 0×, 1× and 2× groups were 15, 38 and 84, respectively. The differences between the 0× and the other two groups are significant at $P<0.001$ using a Student's *t*-test.

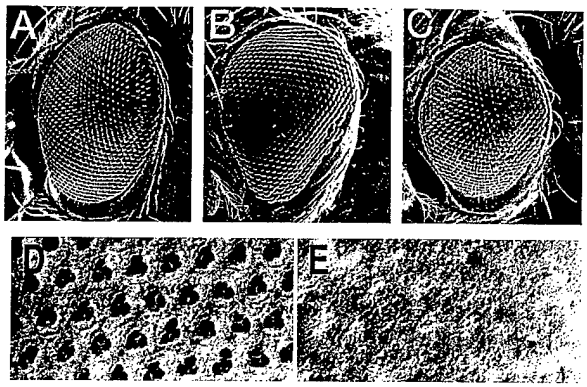

Fig. 6. Overexpression of *dsh* can inhibit bristle formation independently of *wg*. (A-C), SEM micrographs of P[*hs-dsh*] eyes given no heat shock (A) or a 30 minute heat shock (37°C) at 6 hours APF (B) or 9 hours APF (C). When *dsh* was induced at 6 hours APF, more than half the eyes had no or only a few bristles in the center of the eye (n=11) and the rest had a small patch of bristles in the center (n=8). At 9 hours APF, bristles were found over the interior two thirds of the eye but bristles were still missing toward the periphery (n=17). (D,E), cut stainings of P[*hs-dsh*], wg^{IL} homozygotes that were raised at 17°C (the permissive temperature) and then incubated at 29°C for 0-12 hours APF, without (D) or with (E) a 30 minute heat shock at 6 hours APF. Antibody stainings were done at ~30h APF. The cut-positive SOPs (now at the 4-cell stage) are completely absent in the heat shocked eyes (n=8). wg^{IL} homozygotes were identified as described in Materials and Methods.

through *N* or in a parallel pathway converging at proneural gene expression.

Role of *N* in *wg* signaling in the embryo

In order to more rigorously test the requirement of *N* for *wg* signaling, a tissue is needed where a putative *N-wg* connection can be separated from the *wg*-independent functions of *N*. One suitable place is the embryonic epidermis. Embryos mutant for *N* undergo a dramatic neural hyperplasia; almost all of the cells of the epidermis delaminate and become neuroblasts (Campos-Ortega, 1993). However, the epidermis remains relatively intact until full germ-band extension, after significant *wg* signaling has already occurred. Null *N* embryos were generated by making germ-line clones (Chou and Perrimon, 1992; see Materials and Methods). Antibody staining revealed no detectable N protein in *N* germline clones that have received a paternal Y chromosome (Fig. 8F). Thus we can examine *wg* signaling in a tissue that has never contained N protein.

Two well-characterized targets of *wg* signaling in the embryo are the *engrailed* (*en*) gene (DiNardo et al., 1988; Martinez-Arias et al., 1988) and the *wg* gene itself (Bejsovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995). Careful analysis of expression of both genes has revealed that, in *wg* mutants, *wg* transcripts begin to fade before the embryo reaches full germ-band extension (stage 9; all stages according to (Campos-Ortega and Hartenstein, 1985), and is gone by the beginning of stage 10 (Manoukian et al., 1995). en protein in the adjacent posterior cells fades shortly thereafter. By mid-stage 10, both en protein and *wg* transcripts are completely gone from wg^{IN} homozygous embryos (Fig. 8B). In *N* null embryos at early stage 10, *wg* and *en* patterns are indistinguishable from wild type (data not shown). At mid-stage 10, both sets of stripes are still clearly present (Fig. 8C,D). The stripes do appear a little ragged, and we believe this is a consequence of the beginning of the disintegration of the epidermis, which is well underway by late stage 10 (about 15-20 minutes later than the embryos shown in Fig. 8).

Despite the results in Fig. 8, it might be argued that in *N* mutants, perhaps *wg* and *en* expression no longer depended on *wg* activity. To address this, we examined the affect of global *wg* expression on *en* transcript distribution in a *N* mutant background. As previously reported (Noordermeer et al., 1992, 1994), overexpression of *wg* via a heat-shock promoter in an otherwise wild-type background causes a dramatic posterior expansion of the *en* stripes so that they are about twice as wide as normal (compare Fig. 9A and B). This expansion in still seen in embryos lacking N protein (Fig. 9D) and is dependent on the presence of the P[*hs-wg*] transgene (Fig. 9C). In the complete absence of N protein, *wg* signaling appears normal as late as we can reliably assay for it.

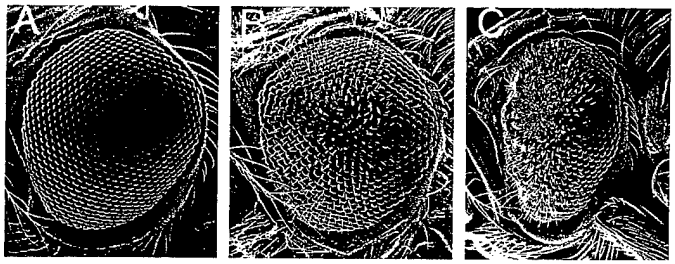

Fig. 7. Removal of *N* or *Dl* activity can suppress the P[*sev-wg*] bristleless phenotype. SEM micrographs of P[*sev-wg*]/+ (A), N^{ts1}/Y; P[*sev-wg*]/+ (B) and P[*sev-wg*], Dl^{RF}/Dl^{6E} (C) flies that were reared at 17°C and incubated at 32°C for 3 to 11 hours APF (7 hours APF at 17°C corresponds to 3 hours APF at 25°C) and then kept at 17°C until eclosion or dissection of pharates from pupal cases. Control and N^{ts1} hemizygotes were made by crossing P[*sev-wg*] males to either *w* or *w* N^{ts1} females. All males then had the desired genotype. P[*sev-wg*], Dl^{RF}/TM6C and Dl^{6E}/TM6C flies were crossed and appropriate animals identified by the absence of the dominant Tubby marker (found on TM6C). All N^{ts1} hemizygotes (n=20) and Dl^{RF}/Dl^{6E} transheterozygotes (n=9) showed the dramatic increase in bristle number. Note the higher than normal bristle density, indicative of the role these genes play in lateral inhibition. The *Dl* mutant combination consistently gave a more severe bristle hyperplasia than N^{ts1} in both a P[*sev-wg*] and non-transgenic background.

DISCUSSION

wg inhibits SOP formation at the level of the proneural genes

The interommatidial bristle is a 4-cell sensory organ that arises from a single SOP which is selected from a group of cells expressing proneural basic helix-loop-helix proteins (Campuzano and Modolell, 1992; Jan and Jan, 1993a). Our data strongly suggests that P[*sev-wg*]-derived wg protein blocks SOP formation in the eye by inhibiting proneural gene expression. Levels of ac protein are much lower in P[*sev-wg*] eyes (at 3 hours APF) compared to controls (Fig. 2C-F). 12 hours later, after the eye disc has everted, no SOP daughter cells are seen in the transgenic eyes (Fig. 2A,B). Though disc eversion prevents us from directly showing that no SOPs ever form in P[*sev-wg*] eyes, the time window when P[*hs-wg*] or P[*hs-dsh*] can inhibit bristle formation (no later than 6 hours APF for the central portion of the eye; Fig. 6 and results) is consistent with the model that, once an SOP is determined, *wg* signaling activity can no longer influence its fate.

The ac protein is the only proneural gene product monitored in this study and we are by no means suggesting that the *wg* signaling pathway acts directly on the *ac* promoter. In fact, lost of the *ac* gene alone does not result in complete elimination of interommatidial bristles; a related gene, *scute* (*sc*) must also be removed (Brown et al., 1991). The expression patterns of *ac* and *sc* are nearly identical (Cubas et al., 1991; Skeath and Carroll, 1991). This is most likely achieved by a combination of shared enhancer elements (Gómez-Skarmeta et al., 1995) and auto- and transactivation between the two genes (Martinez and Modolell, 1991; Skeath and Carroll, 1991; Van Doren et al., 1992). In addition, there are important negative inputs from other bHLH proteins such as *extramacrocheate* (Cubas and Modolell, 1992; Van Doren et al., 1992) and *hairy* (Brown et al., 1991; Van Doren et al., 1994). *wg* could be acting to inhibit *ac* (and presumably *sc*) expression at any of these regulatory levels. Further studies are needed to address this issue.

The P[*sev-wg*] bristleless phenotype was unexpected, because in the wing imaginal disc, *wg* has been shown to have the opposite effect, i.e., it promotes bristle development. In the absence of *wg* activity, the proneural ac-positive clusters fail to form (Couso et al., 1994; Phillips and Whittle, 1993). It is not clear why *wg* activates *ac* in one tissue and inhibits it in another, but this is a simple example of how one signal can generate different responses in various tissues.

wg is not normally expressed in the interior of the eye, but it is present at the periphery, forming a ring around the pupal eye (Cadigan and Nusse, unpublished data). Interestingly, the edge of the eye lacks bristles (Cagan and Ready, 1989b; Fig. 1A). Clones of *arm* at the periphery contain ectopic bristles (Cadigan and Nusse, unpublished data), suggesting that *wg* normally inhibits bristles there. However, large *wg* clones do not show this effect. We are currently examining this in more detail.

The *wg* signal transduction pathway in the eye

A genetic pathway for *wg* signal transduction has been elucidated in which the gene products work in the following order: *porc* → *wg* → *dsh* —| *zw3* —| *arm* (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Studies in the wing and leg imaginal disc have indicated that *dsh*, *zw3* and *arm* are also required there for *wg* signaling (Couso et al., 1994; Diaz-Benjumea and Cohen, 1994; Klingensmith et al., 1994; Peifer et al., 1991; Theisen et al., 1994; Wilder and Perrimon, 1995). This study extends these findings; *porc*, *dsh* and *arm* are clearly required for the ability of *wg* to inhibit eye bristles (Fig. 4; Table 1). The overexpression experiments with *zw3*, while not as conclusive (Fig. 5), are entirely consistent with the favored model, where *wg* acts by antagonizing *zw3* gene activity. While there may be exceptions (see below), it seems that most tissues use the same *wg* signaling components to achieve a variety of effects.

The mammalian counterpart of *zw3*, glycogen synthase kinase-3, has been shown to function in ras-dependent signaling (Stambolic and Woodgett, 1994). This raises the possibility that members of the *ras* and *wg* pathways share components in flies. In the eye, differentiation of photoreceptor cells is absolutely dependent on *ras*-dependent signaling (Simon et al., 1991). However, in clones of *dsh* and *arm*, all photoreceptors are present (S. Kaech, K.M. Cadigan and R. Nusse, unpublished observations). In the wing, clonal analysis with members of the *ras* pathway demonstrated that, unlike *wg*, they were not required for wing margin development (Diaz-Benjumea and Hafen, 1994). Thus, no interaction between these two pathways has yet been observed in *Drosophila*.

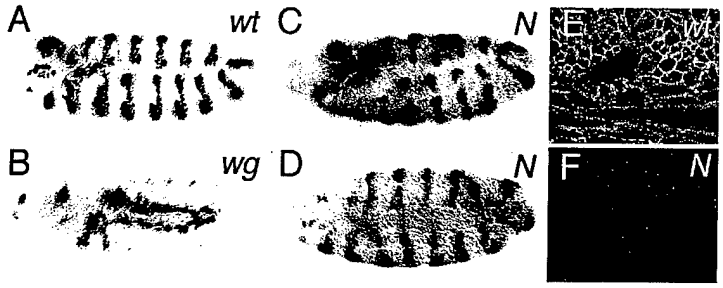

Fig. 8. *wg* signaling appears to be normal in *N* null mutant embryos. (A-D) Whole-mount staining for *wg* transcripts (blue) and/or en protein (brown) in wild-type (A), wg^{IN} (B) or N^{5419} (C,D) mutant embryos. All embryos are at mid-stage 10 (Campos-Ortega and Hartenstein, 1985). Both *wg* and *en* are absent at this stage from the epidermis of the *wg* mutants, but remain robust in the *N* mutant background (these embryos were also stained for β-gal protein, to unambiguously identify maternal and zygotic *N* mutants (see Material and Methods). (E,F) Confocal images of N antibody staining with a monoclonal antibody directed against the intracellular domain of N (Fehon et al., 1990) in N^{5419} germ-line clones receiving a paternal P[*ftz-lacZ*] (E) or Y (F) chromosome. N signal is completely lacking in the embryos that are negative for β-gal protein. Similar results in *wg*, *en* and *N* expression were obtained with a second *N* null allele, $N^{264.40}$ (data not shown).

(Fig. 8A-D are a little less in contrast than original)

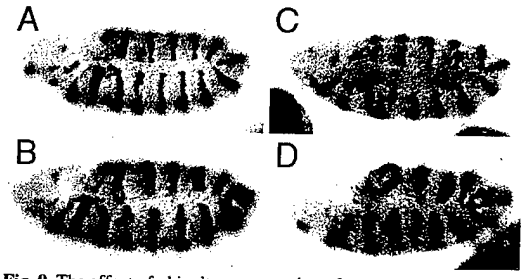

Fig. 9. The effect of ubiquitous expression of *wg* on *en* transcript distribution is still seen in a *N* null mutant background. All embryos are whole-mount stainings of *en* transcripts. (A) P[*hs-wg*] embryo with no heat shock. The *en* stripes are normal in appearance. (B) P[*hs-wg*] embryo after three 20 minute heat shocks (37°C) during early embryogenesis. The *en* stripes have expanded posteriorly, to about twice their normal width as previously described (Noordermeer et al., 1992). (C) N^{5419} null mutant after the three heat shocks. The stripes are somewhat ragged, but still present at the normal width. (D) N^{5419}; P[*hs-wg*] embryo after heat-shock treatment. The stripes have broadened as they do in a N^{+} background. All embryos were mid-stage 10 and the same results were obtained using the $N^{264.40}$ allele. *N* null embryos were created and identified as described in Materials and Methods.

wg expression is subject to positive autoregulation in the embryo (Bejsovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995) and recent evidence suggests that this occurs through a distinct signaling mechanism (Hooper, 1994; Manoukian et al., 1995). Some discrepancies exist between the two reports, but Manoukian et al. (1995) provide strong evidence that *wg* autoregulation requires *porc* but not *dsh*, *zw3* and *arm*. They suggest a model where *porc* functions only in *wg* autoregulation and the other three genes in *wg* paracrine functions.

Our results in the eye indicate that, at least in the eye, *porc* is required for *wg* paracrine signaling. While we could clearly see *sev* enhancer-driven *wg* expression in cone cells and photoreceptors, we found no expression in the proneural clusters, the targets of *wg* action (Fig. 2). The endogenous *wg* gene was not required for the P[*sev-wg*]-dependent bristle inhibition (Fig. 4C), ruling out a paracrine-autocrine circuit. Our results indicating a role for *porc* in paracrine *wg* signaling are consistent with the observation that secretion or diffusion of wg protein is blocked in porc mutant embryos (Siegfried et al., 1994; van den Heuvel et al., 1993a).

Overexpression of *dsh* can mimic the action of *wg* in the eye (Fig. 6) as has been shown previously in the wing (Axelrod et al., 1996) and in cultured cells (Yanagawa et al., 1995). In the wing, this effect of *dsh* required *wg*. This does not appear to be the case in the eye (Fig. 6E). This is an important point because it speaks as to whether *dsh* can completely bypass the requirement for *wg* or whether overexpression of *dsh* simply potentiates *wg* signaling. It may be that there is residual *wg* activity left in our experiments (we could only rear the animals for 6 hours at the restrictive temperature before induction of *dsh*; longer times killed the organism before disc eversion). Another possibility is that a much higher threshold of *wg* activity is needed to transform wing blade to wing margin than is needed to inhibit eye bristles. The data of Axelrod et al. (1996) show that the transformation of identity is more penetrant closest to the normal wing margin, where *wg* is expressed. Thus, overexpression of *dsh* in the wing blade may not easily reach the necessary level of signaling to trigger the change in cell fate. In the eye, *dsh* is able (at 3 hours APF) to inhibit bristles in the middle of the eye (far from endogenous *wg* expression) just as efficiently as bristles closer to the periphery. That *dsh* can bypass the need for *wg* is also supported by the cell culture experiments (Yanagawa et al., 1995) where no detectable wg protein was observed under conditions where *dsh* could stabilize arm protein. In addition, Park et al. (1996) have recently shown that overexpression of dsh in the embryo can induce wg targets in a wg null background.

Is *N* required for *wg* signaling?

On the basis of genetic interactions between mutations in the two genes, the N protein was proposed to be a receptor (or part of a receptor complex) for wg (Couso and Martinez Arias, 1994). In the eye, we also observed strong genetic interactions between *wg* and *N* (Fig. 7). However, the interpretation of these experiments are complicated, since *N* is known to affect bristle development independently of *wg*, and because, for technical reasons, we could not completely remove *N* activity to determine whether *wg* signaling could still occur. Likewise, the previously published genetic interactions involve animals where *wg* and *N* activities are only partially removed (many of the experiments were done with double heterozygotes of various *wg* and *N* alleles), and are therefore subject to the same limits of interpretation.

Unlike the eye, *wg* signaling in the complete absence of *N* activity can be assayed in the embryonic epidermis until just after germ-band extension is complete (mid stage 10), right before the absence of *N* causes most of the epidermis to delaminate and become neuroblasts. We found no significant change in the expression of *wg* and *en* in *N* null mutants at this time (Fig. 8), even though their expression fades at early stage 10 in *wg* mutants and mutants in *dsh* or *arm* (Manoukian et al., 1995; Van den Heuvel et al., 1993b). In addition, the effect of overexpression of *wg* on the *en* stripes is still seen in a *N* mutant background (Fig. 9). Couso and Martinez Arias (1994) reported that the *en* stripes were affected in about half the *N* mutants they examined, but they used hyperplasia of the nervous system as their method for determining which embryos were *N* mutants. This happens after mid-stage 10, thus any effect on the stripes may be a secondary consequence of the epidermis falling apart. Therefore, we conclude that in *N* mutant embryos, *wg* signaling occurs normally, at least with regard to the two markers we assayed.

A similar conclusion with regards to *N-wg* interactions has been reached in the wing (Rulifson and Blair, 1995). They showed that *wg* could still regulate *ac* expression in homozygous clones for a *N* null allele. These mutant clones should completely lack *N*, barring prolonged perdurance of the N protein. Of equal importance is their finding that *N* activity is required for *wg* expression at the wing margin (see also Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). This means that all of the genetic interactions between *wg* and *N* in the wing can potentially be explained by a reduction in *N* activity causing a reduction in the amount of *wg* signal, not the ability of *wg* to signal.

Another link between *wg* and *N* has been proposed by Axelrod et al. (1996), who have presented evidence that dsh protein can bind to and inhibit N activity in the wing imaginal disc. They suggest that part of the ability of *wg* to induce bristles in the wing is achieved by inhibition of *N* through *dsh*. Such an antagonistic relationship does not appear to be occuring in the eye since *wg*, *dsh* and *N* all inhibit bristle formation, although we can not rule out a mechanism where *wg* and *dsh* activate *N* to inhibit *ac* expression.

A subtle role for *N* in transducing the *wg* signal cannot be entirely ruled out. However, our results and those of Rulifson and Blair (1995) argue that in tissues where the direct test can be done, i.e., can *wg* signaling occur in cells that lack N protein, *N* is not required. A better candidate for a *wg* receptor is the product of the *Drosophila frizzled2* gene, which can bind to wg and tranduce the *wg* signal in cultured cells (Bhanot et al. 1996). N showed no activity in this wg-binding assay. In the absence of any biochemical data suggesting that the proteins interact, the simplest models for *wg* signal transduction should exclude a direct role for *N*.

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